Software Manual

ZEISS ZEN 3.3 (blue edition)

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Original Manual

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1 Welcome

ZEN (blue edition) is a modular image acquisition, processing and analysis software for digital microscopy. The abbreviation ZEN stands for ZEISS Efficient Navigation and points out that the software can be used to control microscopes and imaging systems by ZEISS.

In addition to basic functionality for image acquisition, elementary image processing and annotations and image analysis a lot of optional modules for specific microscopy tasks are available.

With ZEN lite the basic version of the software is available for free. Starting from a basic functionality for image acquisition, simple image processing, image analysis and documentation a lot of optional modules are available for ZEN lite as well. More detailed information is available in the product brochure.

2 General Information

2.1 Using the ZEISS Help Viewer

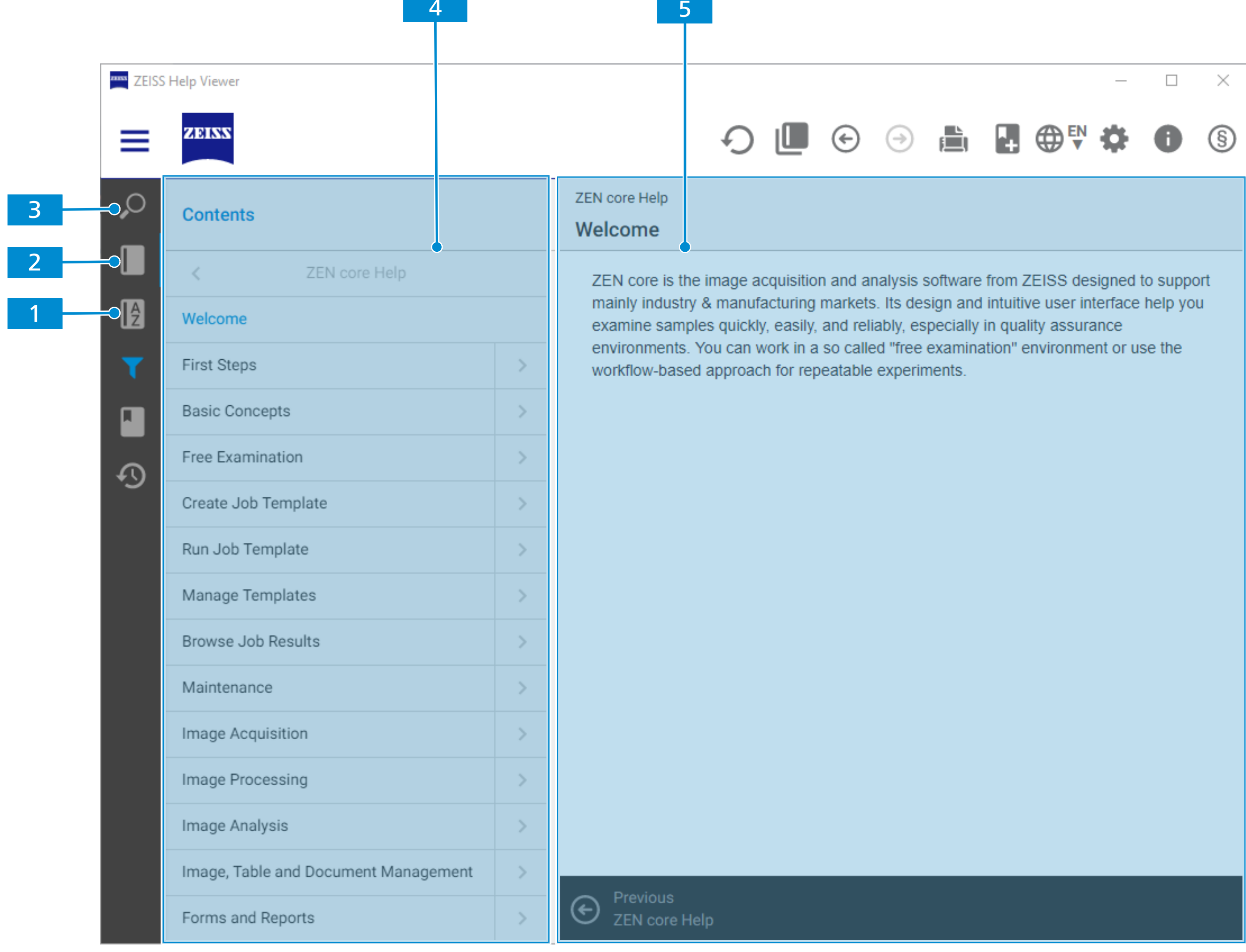
Opening the ZEISS Help Viewer

If you need help on a specific area, tool, or section within the software, simply press the [F1] key to open the related help topic.

As it is difficult sometimes for the software to know on which topic you want help for when pressing the [F1] key, you can use the **Question Mark** symbol alternatively. Simply click on the question mark symbol **?** in the **Title bar**. The courser then appears as a question mark symbol. Click on an area in the software for which you want to get help. If there is a related help topic available it will open directly.

ZEISS Help Viewer User Interface

The following screenshot indicates the main elements of the user interface:



1 **Index**

List of keywords to help you find topics and content quickly

2 **Topics**

Contains the structure tree with a list of all the topics.

3 **Search**

Search through the entire text
It supports partial strings but not wildcards.

4 **Structure tree**

Enables you to navigate through topics sequentially. A **>** indicates a topic has subtopics.

5 **Content panel**

2.2 Safety Notes and Safety Labels

The display of safety notes in the documentation and software follows a system of risk levels, that are defined as follows:

⚠ CAUTION

Risk of personal injury

CAUTION indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate personal injury.

NOTICE

Risk of property damage

NOTICE indicates a property damage message. In addition, NOTICE is used for data loss or corrupt data as well.

The safety icons / labels on the device or in the documentation refer to potential dangers or information that are defined as follows:

Icon / Label	Name	Description
	Crushing Fingers	This icon warns you of a potential risk of crushing fingers.

2.3 Text Conventions

The following text conventions are used in this documentation:

Format	Description
Format "**bold**"	The format "Bold" within text is used for ▪ Clickable user interface elements, e.g. buttons and icons **Example**: Click on **Save**. ▪ Hardware buttons on the microscope **Example**: **On/Off** button ▪ Non-clickable user interface elements, e.g. name of a dialog **Example: Image** dialog

Format	Description
Format "*italic*"	Italics highlights the following: ▪ text to be entered by the operator
Format for keyboard text	Text in bold + brackets is used for keyboard commands. E.g. **Example:** Press **[F1]** to open the online help.
Format for a path in the software	Description of a path within the software. **Example:** Go to **Tools > Options > Acquisition**
Format for programming code	Used for programming code, e.g. macro code as well as for anything that you would type literally when programming, including keywords, data types, constants, method names, variables, class names, and interface names. **Example**: `Integer`
Format for links (internal or web links)	Links to further information internally or in the web. **Example**: Link
Format for procedural instructions	A numbered procedural instruction is used for actions which are performed by the user. The steps must be performed in the given order. Optionally there are pre-requisites which have to be fulfilled in advance. At the end of the instruction normally the result of the procedure is presented. **Example:** 1. Action step which has to be actively performed by the user → Intermediate result 2. Another step within the procedure.

Additional information is indicated as follows:

Info

Helpful additional information, e.g. about necessary additional actions.

2.4 Legal Notes

ZEISS draws the User's attention to the fact that the information and references contained in these documentation may be subject to technical modifications, in particular due to the continuous further development of ZEISS products. The documentation enclosed does not contain any warranty by ZEISS with regard to the technical processes described in the documentation or to certain reproduced product characteristics. Furthermore, ZEISS shall not be held liable for any possible printing errors or other inaccuracies in this documentation, unless proof can be furnished that any such errors or inaccuracies are already known by ZEISS or that these are not known to ZEISS due to gross negligence and that furthermore ZEISS has for these reasons refrained from eliminating these errors or inaccuracies appropriately. ZEISS hereby explicitly draws the User's attention to the fact that this documentation only contains a general description of the technical

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ZEISS explicitly draws attention to the fact that the information contained in this documentation will be updated regularly in compliance with the technical modifications and supplements carried out in the products and furthermore that this documentation only reflects the technical status of ZEISS products at the time of printing.

Safety

Refer to the safety notes and instructions in the documentation of all necessary devices (e.g. microscope peripherals, cameras, computers, computer accessories, etc.) before installing and using the software.

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Notice of the Producer

This software product was designed, realized, verified, validated and released in a certificated process environment. The quality management system is certified following the rule of DIN EN ISO 13485.

The fields of application of the Software are common tasks and applications in microscopy respectively imaging (so called “Off-The-Shelf Software”). Though the user acknowledges that in any kind of use the end user of the software is responsible for the validation of the Software for the end user’s dedicated intend of use considering all requirements of law and standards (e.g. FDA/21 CFR part 11, IvDD, etc.). If necessary the end user has to establish, to document, to implement and to maintain a special process to fulfill all the requirements to be conform with the validate rules of law and standards. It is pointed out that displayed measure values (e.g. length measurement) may not be used directly as analytical values for diagnostic results.

CARL ZEISS DOES NOT WARRANT THAT THIS SOFTWARE IS USABLE FOR SPECIAL PURPOSES OTHER THAN IN THE FIELDS OF APPLICATION DEFINED ABOVE.

3 First Steps

3.1 Starting the Software

Prerequisite ✓ You have installed ZEN (blue edition) on your computer.

1. Double click on the program icon on your desktop.

2. Alternatively click on **Start** > **All Programs** > **Carl Zeiss Microscopy** > **ZEN** > **ZEN (blue edition)** entry (blue icon).
   → The software starts. After a while you see the login screen.

3. Click on the button of the application you want to work with. The available applications depend on your licenses and system (e.g. if you work with an LSM, only **ZEN system** and **Image Processing** is available). Make sure that the hardware components you use are switched on and are ready for operation.
   → The software starts. During the program start the hardware settings will be initialized.

You successfully started the software.

Info

For using pre-recorded images when starting the software, in the menu **Tools** > **Options** > **Startup**, the **Reload Last Used Documents** checkbox must be activated.

3.2 User Interface

The software user interface is divided into three main areas.

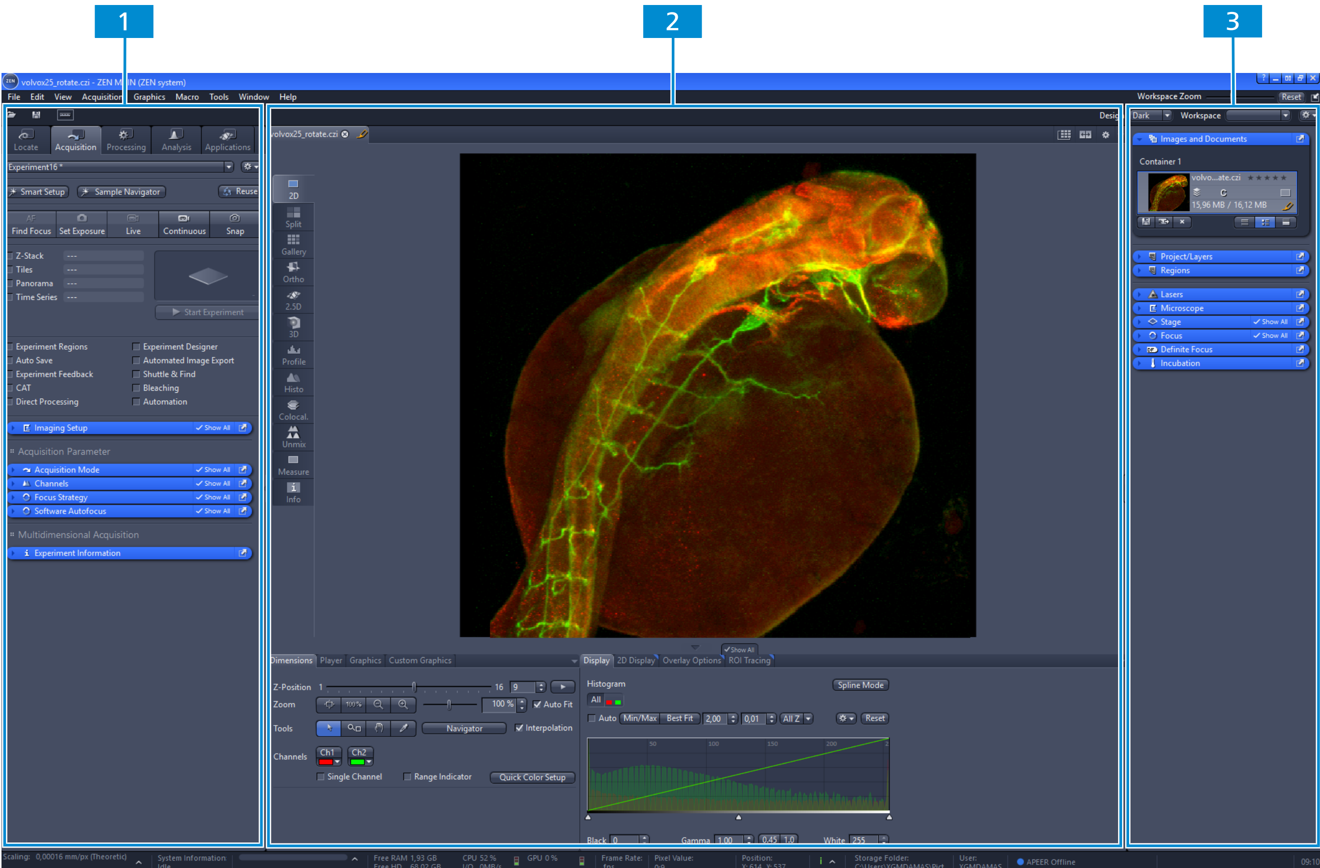


Fig. 1: User interface

1 **Left Tool Area**
With the tabs you can access all the main tools for microscope control (*Locate Tab [▸ 648]*), acquisition (*Acquisition Tab [▸ 652]*), image processing (*Processing Tab [▸ 664]*), image analysis (*Analysis Tab [▸ 665]*), and applications (*Applications Tab [▸ 665]*).

2 **Center Screen Area**
This area is used to display your images with several image views available. For more information, see *Center Screen Area [▸ 21]*.

3 **Right Tool Area**
Here you find the *Images and Documents Tool [▸ 797]*, the Objective Selection and the Stage and Focus controls. Additionally system specific tools can be available here (e.g. **Definite Focus** and **ZEN Connect** controls).

3.2.1 Title bar

Parameter	Description
Help icon	Activates the "drag & drop" help function. A question mark appears beside the mouse pointer. Move the mouse pointer to a place in the software where you need help. Left-click on the desired location. The online help opens.
Minimize	Minimizes the program window.

Parameter	Description
Maximize Over 2 Screens	Maximizes the program window across 2 screens if available. This option is only possible if you are working with 2 screens with the same resolution.
Maximize	Maximizes the program window to the main screen.
Restore Down	Reduces the program window to any selected size.
Close	Closes the program window.

3.2.2 Workspace Configuration

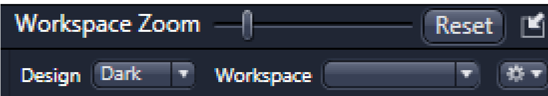


Fig. 2: Workspace Configuration

Here you find settings to adjust your workspace. Select **Light/Dark Design** of the user interface or enlarge the screen with **Workspace Zoom** slider. You can also save and reload all personal settings in a workspace configuration. With the **Dock all tool windows** button in the top right corner you can easily dock all undocked tools by one click.

3.2.3 Menu bar

Fig. 3: Menu bar

The menu bar contains all the menus you need for managing, editing, and viewing your projects.

See also

- File Menu [▸ 613]
- Edit Menu [▸ 617]
- View Menu [▸ 618]
- Acquisition Menu [▸ 619]
- Graphics Menu [▸ 620]
- Macro Menu [▸ 622]
- Tools Menu [▸ 626]
- Window Menu [▸ 646]
- Help Menu [▸ 647]

3.2.4 Tool bar

Fig. 4: Tool Bar

Here you gain quick access to important functions, e.g. saving or opening files. Further right you find more workspace settings, e.g. **Design** and **Workspace** selection. Read how to customize the Tool bar in chapter *Customizing Toolbar [▸ 237]*.

3.2.5 Left Tool Area

This area contains the main tabs for microscope and camera settings (**Locate** tab), image acquisition (**Acquisition** tab), image processing (**Processing** tab), and image analysis (**Analysis** tab). The main tabs are organized in an order which follows the typical workflow of experiments in bioscience or material science.

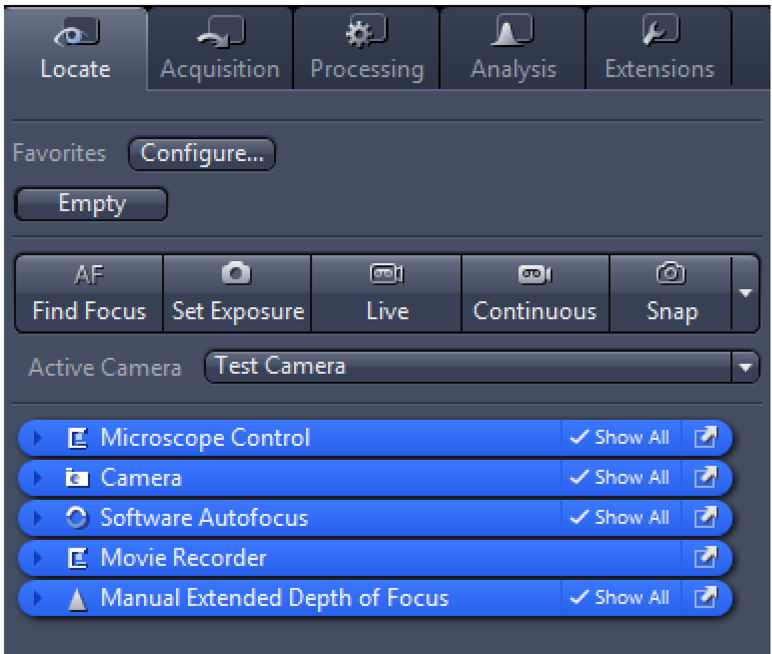


Fig. 5: Left Tool Area

See also

- Locate Tab [▸ 648]
- Acquisition Tab [▸ 652]
- Processing Tab [▸ 664]
- Analysis Tab [▸ 665]
- Extensions Tab [▸ 666]

3.2.6 Center Screen Area

The Center Screen Area is structured in 4 areas.

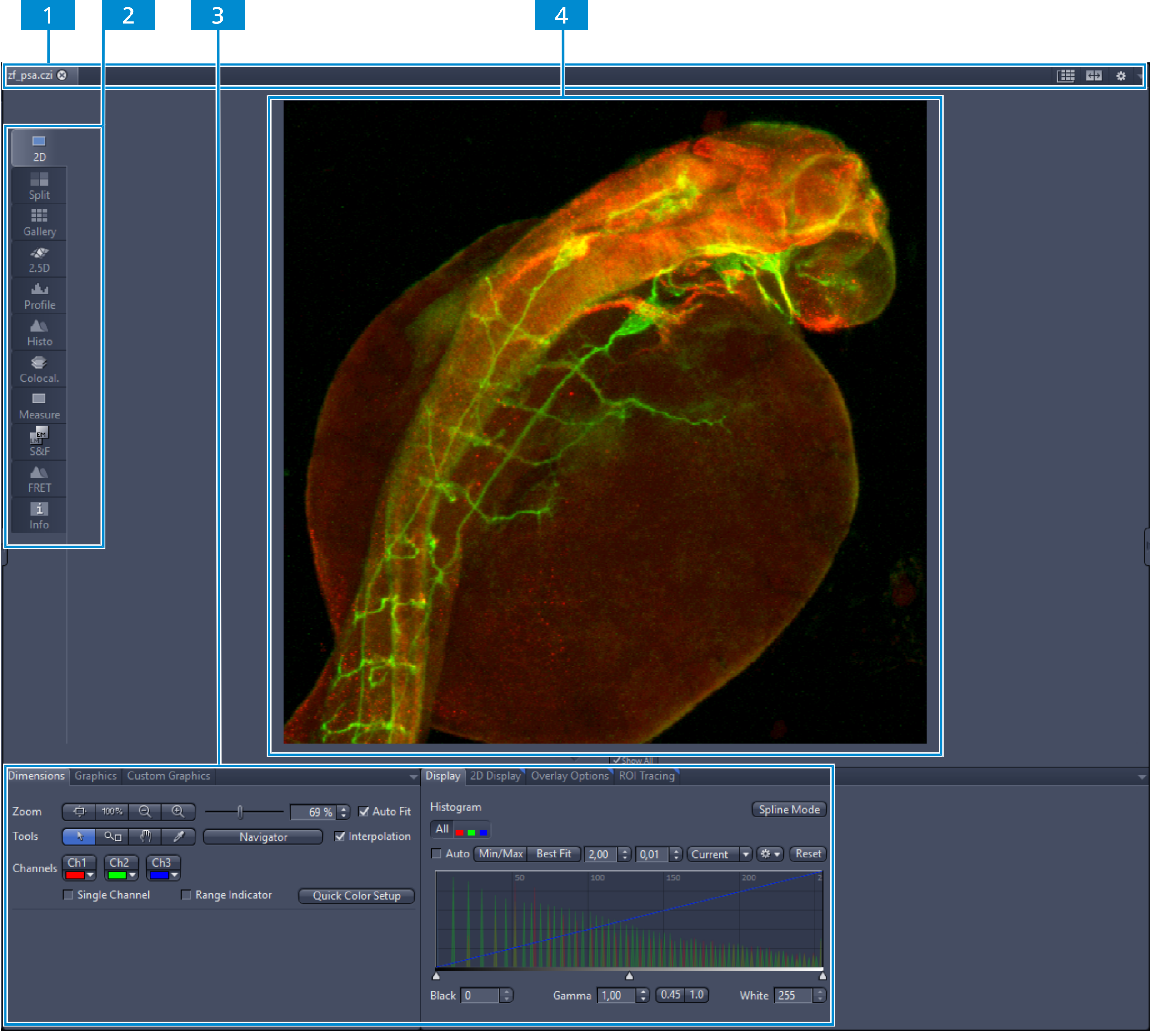


Fig. 6: Center Screen Area

1 **Document bar**
Here you can switch between all your opened documents. For more information, see *Document bar [▸ 22]*.

2 **Image Views**
Area where you can switch between different image views by selecting the corresponding tab in the list. For more information, see also *Image views [▸ 810]*.

3 **View Options**
Area for general and specific view options. For more information, see also *General View Options [▸ 887]*.

4 **Image Area**
Area where images, reports, and tables are displayed.

3.2.7 Right Tool Area

This area contains mainly the tools for image and file handling (e.g. **Image Gallery**) and hardware control (e.g. **Stage** / **Focus** tool). Depending on your system configuration, other tools can be available. The tools are described in the corresponding chapters of the online help.

See also

- ZEN Connect Tool [▸ 601]
- Stage Tool [▸ 798]
- Focus Tool [▸ 800]
- Incubation Tool [▸ 803]
- Microscope Tool [▸ 798]
- Images and Documents Tool [▸ 797]
- Macro Tool [▸ 804]

3.2.8 Document bar

Here you see tabs of all open documents. Click on a tab to view the image/document. On the right end of document bar you find buttons to switch view mode from **Exposé** to **Splitter** mode and further view options (**View** menu).

Info

A **asterisk** (*) next to an image/document title indicates that unsaved changes have been made to this document. Save your pictures/documents from time to time in order to avoid data loss.

Parameter	Description
Exposé Mode	Opens the exposé view mode. For more information, see *Exposé mode [▸ 909]*.
Splitter Mode	Opens the splitter view mode. For more information, see *Splitter mode [▸ 909]*.
Options	Displays the options of the view menu. For more information about the individual options, see *View Menu [▸ 618]*.

3.2.9 Status bar

The status bar shows important information on the system status:

Parameter	Description
Scaling	Displays which lateral scaling is currently being used. If you click on the arrow, the *Scaling dialog [▸ 629]* will be opened. There you have access to advanced scaling settings and the scaling wizard.
System Information	Always shows the latest, currently active process that the system is performing.

Parameter	Description
Progress bar	Displays the progress of the currently active process. Each new process added supersedes older still active processes. If you click on the arrow button, a window opens with a list of all processes in chronological order. You can stop a process that is running using the **Stop** button.
Performance indicators	In this group you will see an overview of the performance of individual computer components: ▪ **Free RAM** indicates how much physical memory is still available. ▪ **Free HD** indicates how much space is still available on the hard drive onto which the next image is to be acquired (see **Tools > Options > Saving**). ▪ **CPU** indicates the usage of the Central Processing Unit. ▪ The small **status bar** provides an overall assessment of the system usage. ▪ **GPU** indicates the usage of the Graphics Processing Unit by ZEN and ZEN related services. It is also visualized by a small status bar on its right. **Note:** This GPU indicator is only visible if you your computer has Microsoft Windows 10 version 1709 or higher. **Info**: Double-clicking in the Performance Indicators area opens the Windows Task Manager.
Frame Rate	Indicates the current frame rate in frames per second (fps) used by the active camera for producing new images. Please note in most cases that at speeds greater than 100 frames per second, this value cannot always be accurately determined.
Pixel Value	Displays the gray value to the image at the current position of the mouse pointer. In the case of multichannel images the gray value/ channel is displayed for up to 4 channels.
Position	Displays the X/Y position (in pixel coordinates) of the mouse pointer in the image.
Information (i)	If you click on the icon, a window opens with a *System Messages [▸ 24]*.
Storage Folder	Displays the location where new images are automatically saved. This path can be changed in the menu **Tools > Options > Saving**.
Status: Airyscan Detector Alignment	If you click on the arrow the **Alignment Tool** window opens. See *Airyscan Detector Adjustment [▸ 980]*.
User	Shows the Windows user name of the logged in user.
Time	Shows the current Windows system time.

3.2.9.1 System Messages

If you right click on a system message the **Copy** button will appear. Left click on **Copy** button to copy the message to clipboard. Then paste it into a text file or an E-Mail. The idea behind is that you can easily send error messages to your support team for example. This copy/paste function works for all upcoming system messages or error messages within the application as well.

Parameter	Description
Information	System information that arises during normal operation. This system information does not lead to an interruption of the workflow. The information window is not displayed automatically.
Warnings	Information that requires input from the user, e.g. a prompt to change a manual microscope component. This information leads to the information window being shown briefly. However, it closes again after a few seconds.
Errors	Error messages indicate a malfunction by the system. In this case the information window opens and remains open. The system requires input from the user in order to continue.

Info

Hundreds of messages can accumulate in the course of a session. A maximum of 300 messages are displayed. To display messages for a certain category, activate or deactivate the corresponding checkboxes.

3.3 Setting the User Language

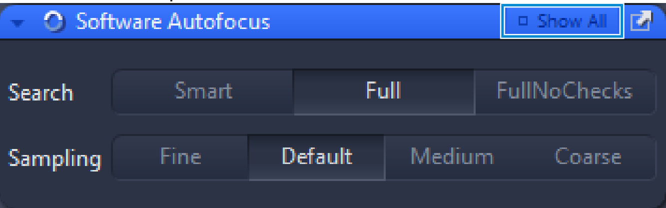
Prerequisite ✓ You have successfully started the application.

1. Click on menu **Tools> Options**.
   → The **Options** dialog opens. The **General** entry in the **Software** group is selected.
2. Deactivate the **Select Automatically** checkbox if you want to set the language manually.
3. Select user language from the **Fixed Language** dropdown list.
   → A message appears to restart the application.
   → Note, that the availability of additional languages can differ between software versions.
4. Click on **OK**.
   → The **Options** dialog closes.
5. Exit and restart software.

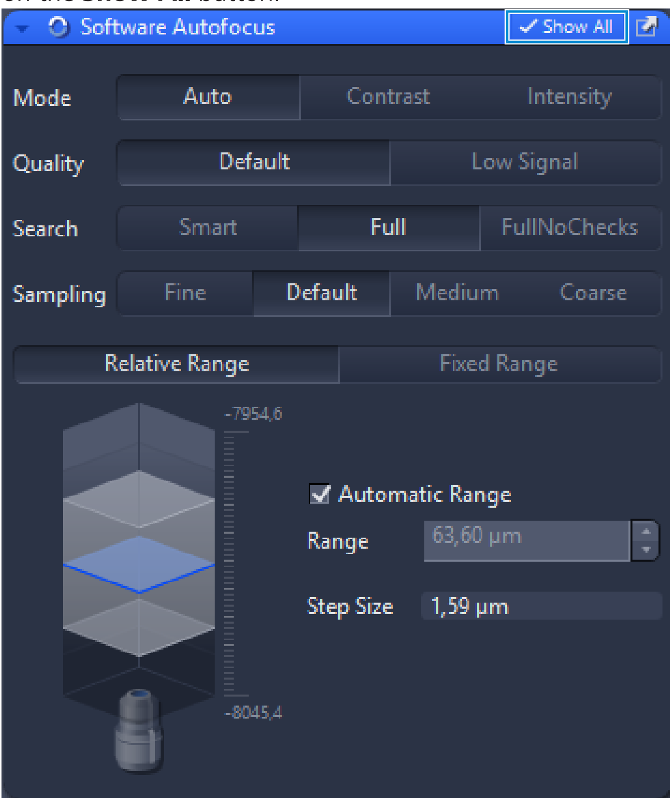
You have successfully set the user language.

3.4 Acitvating the Show All Mode

1. With the **Show All** mode deactivated (default setting), only the basic functions of tool windows or view options are shown.



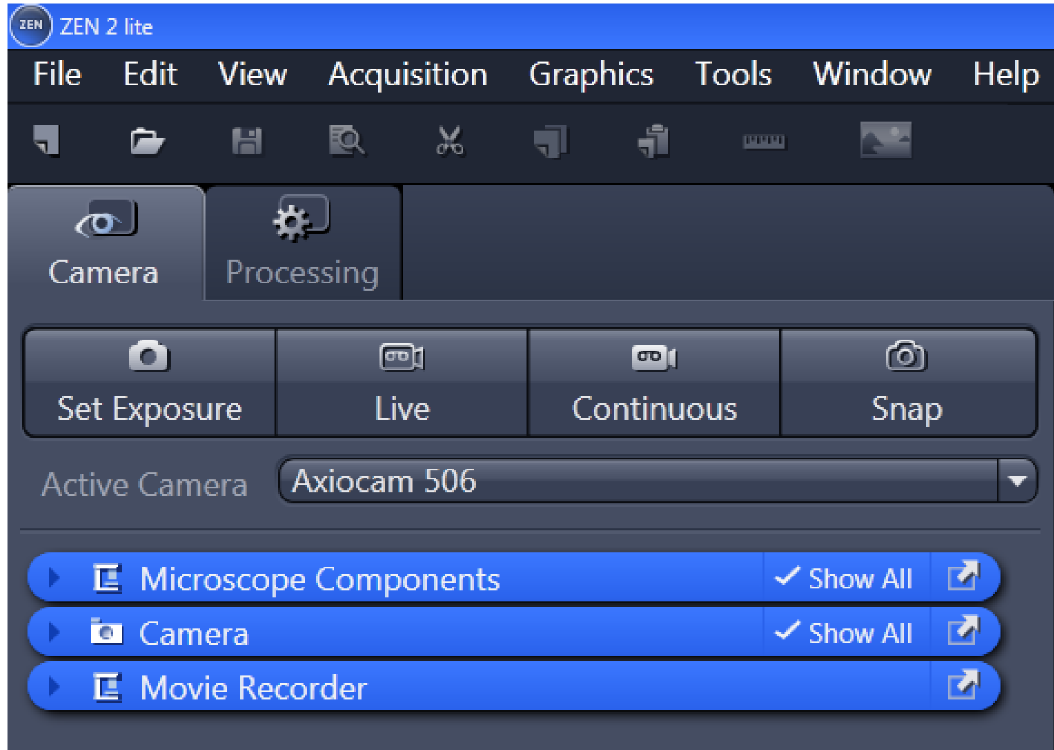
2. To show the advanced settings or expert functions of tool windows or view options, click on the **Show All** button.



3.5 Configuring Microscope Components

This chapter refers to the manual configuration of the microscope components in **ZEN lite**. All microscope components definitions will be stored in the meta data of the acquired image.

Prerequisite ✓ You have selected the **Camera** tab.



1. Click to the **Microscope Components** tool.

→ The tool will open. Consider that the button **Show all** is activated.

2. For **Objective** select that objective you will use for your acquisitions.
3. Select all other microscope components you eventually will use (i.e. Optovar, Reflector, etc.).

You have successfully configured your microscope components.

Info

If you have activated the **Select automatically** button in the status bar under **Scaling** (standard settings), the scaling will be calculated on the basis of your definitions. If you want to perform a manual scaling, read the chapter *Creating a Manual Scaling [▸ 30]*.

See also

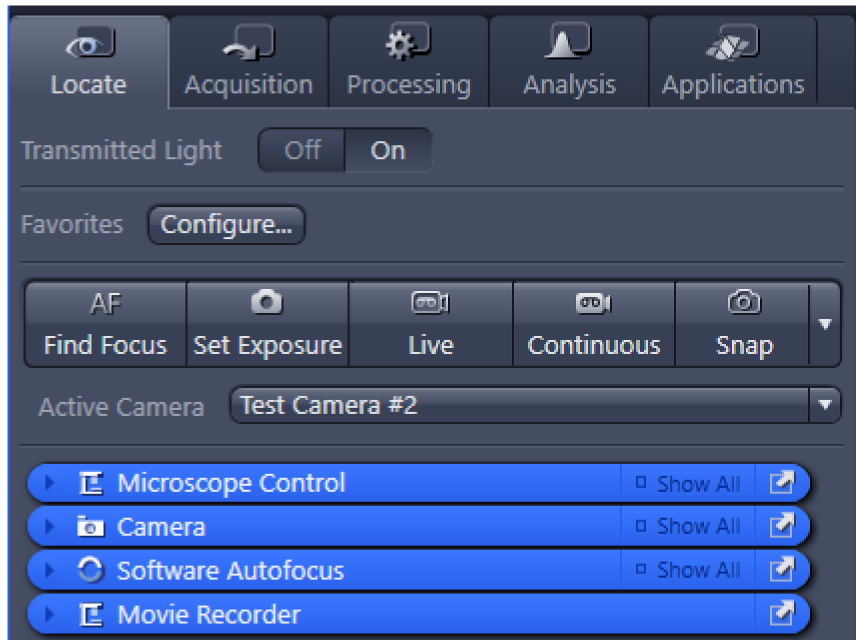
- Creating a Manual Scaling [▸ 30]

3.6 Acquiring a First Camera Image

This topic guides you through acquiring your first camera image with the software.

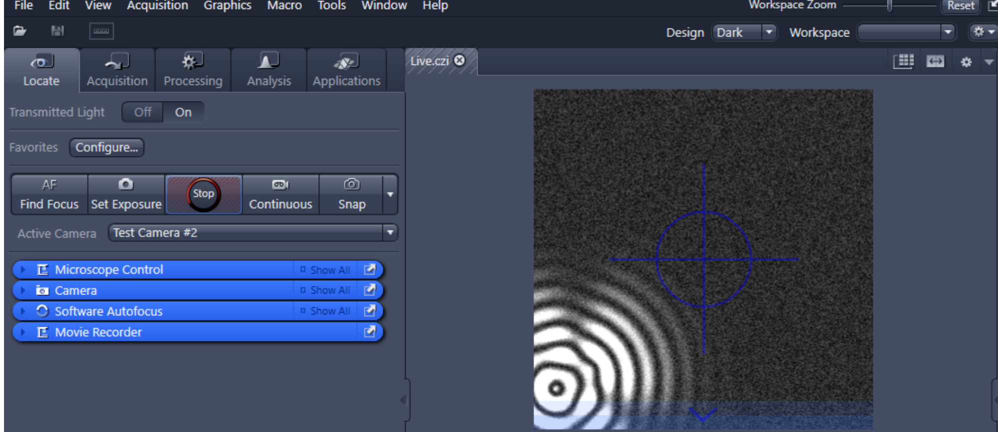
Prerequisite

- ✓ You have connected and configured a microscope camera (i.e. Axiocam 305 color/mono) to your system.
- ✓ You have started the software.
- ✓ You have configured the microscope components (e.g. objective, camera adapter) und you are using the automatic or manual scaling.
- ✓ You are on the **Camera** (ZEN light only) or **Locate** tab.
- ✓ You see your microscope camera available in the **Active Camera** section. If not, select the camera from the list.



1. Position your sample on the microscope and adjust the microscope to see a focused image through the eyepieces.
2. Adjust the tube slider of the microscope to divert the image to the camera (e.g. **50% camera** and **50% eyepieces**).
3. Click on **Live** button.

   → The **Live Mode** will be activated. In the **Center Screen Area** you will see the camera live image. By default the live image shows a cross hair helping to navigate on the specimen. In the chapter *Adjusting Live Image Settings [▸ 30]* you will learn how to optimize live image display.



4. Click on **Set Exposure** button.
   → The exposure time will be automatically determined and set.
5. Click on **Snap** button.

You successfully acquired your first image. Save the image in the file system via the **File** menu | **Save as**.

Info

If you do not see a focused image please refocus the specimen on the microscope. You may activate the focus bar as an additional aid. Right click in the **Center Screen Area** to open the context menu. Select the entry **Focus Bar**. The focus bar will be shown.

See also

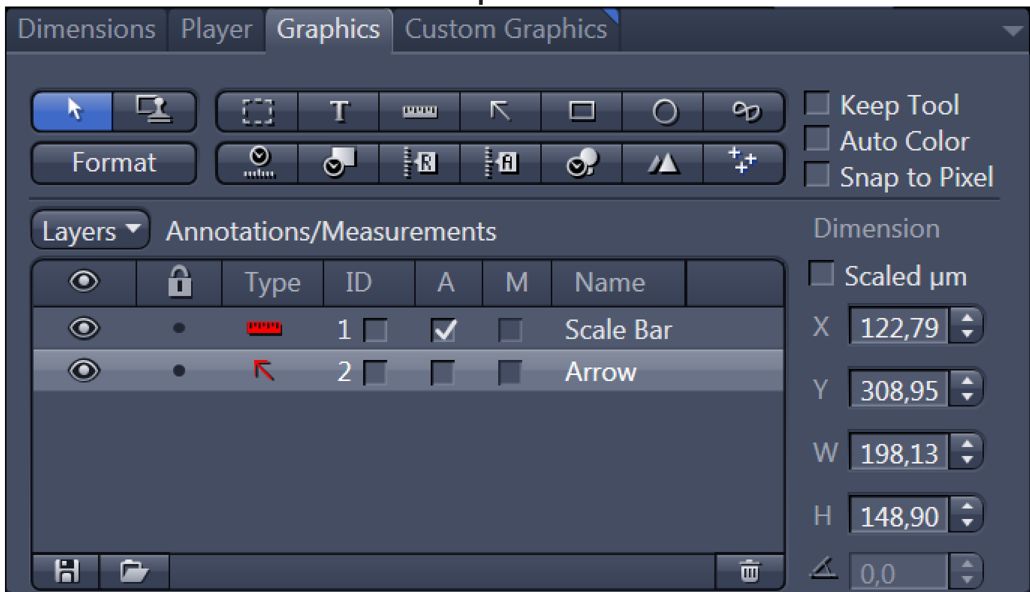
Document bar [▹ 22]

3.7 Adding Annotations to an Image

Annotations are the generic term for all the graphics (e.g. rectangle, arrow, scale), measurements, texts or other metadata (e.g. recording time) that you can add to your image.

Prerequisite ✓ You have acquired or loaded an image.

1. In the **Center Screen Area** select the **Graphics** tab.



2. Click on the Scale Bar button.
   → The scale bar will appear directly in the image.
   → To edit an annotation (e.g. color, line width) you can right click on the annotation in the image and select **Format Graphical Elements** from the context menu.
3. Click on the **Draw Arrow** button.
   → Now you may draw an arrow into your image.

You added the annotations **Scale Bar** and **Arrow** from the toolbar to your image.

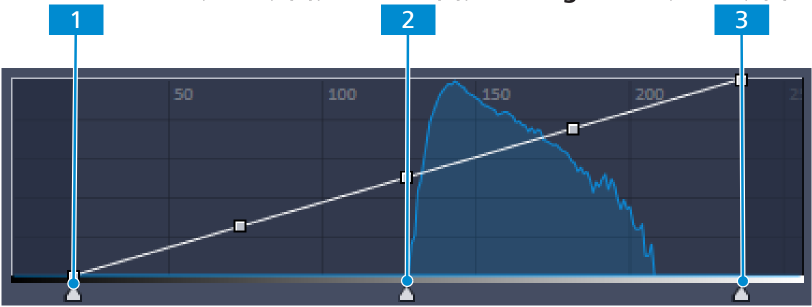
See also

Adding Annotations to Images or Movies [▹ 61]

3.8 Adjusting Live Image Settings

Prerequisite
- ✓ You have started the **Live** mode via the **Live** button and see the camera's live image in the **Center Screen Area**.
- ✓ Under the image area you see the general view options on **Dimensions** tab, **Graphics** tab and **Display** tab.

1. In the **Dimensions** tab activate the **Range Indicator** checkbox. This will mark overexposed (too bright) areas in the live image in red and underexposed (too dark) areas in blue.
2. On the **Display** tab click the **0.45** button. The display curve will be adapted to a gamma value of 0.45. This will set the optimum color presentation. If you do not see this button, activate the **Show all** mode.
3. Move the controls under the display curve left and right in order to directly adjust the values for **Contrast** (**Black**) **(1)**, **Gamma (2)**, and **Brightness** (**White**) **(3)** in the live image.



Info

With the settings above the display of the live image will be adapted. These settings will also be transferred to your acquired image. This will not change the camera settings.

3.9 Creating a Manual Scaling

Prerequisite
- ✓ You have an object micrometer oriented horizontally on the microscope stage.
- ✓ You have selected all definitions for your microscope correctly in the **Microscope Components** tool (ZEN lite only). In our example we use an objective with a 10x magnification.

1. Acquire an image (see *Acquiring a First Camera Image [▸ 28]*) of the scale in your object micrometer using the objective to be scaled manually.
2. In the bottom status bar click on the arrow in the **Scaling** area. In the **Scaling** dialog deactivate the **Select Automatically** checkbox.
3. In the **Create new scaling** section, click on the **Interactive Calibration...** button.
   → The calibration wizard will appear in the image area.
4. Click on single **Reference Line** button (selected as default) and activate the **Automatic Line Detection** button (activated as default).
5. Draw in the reference line along the scale.
6. Enter the true distance between both scale lines in the calibration wizard. In our example this is 500 micrometer.
7. Enter a name for the scaling (i.e. Obj 10x) and click the **Save Scaling** button.

You performed a manual scaling for your objective. Repeat this sequence for all objectives you will need a manual scaling for. Always ensure that you did select the correct objective in the tool **Microscope Components** and for this performed and selected the matching scaling in the status bar.

Info

- The function **Automatic Line Detection** calculates the theoretical maximum of the reference line’s both end points to the closest scale lines in the image. Thus the distance will be calculated with sub-pixel accuracy.
- If you defined manual scalings for your available objectives, and you click in the status bar in the **Scaling** area to open the **Scaling** dialog and to activate the checkbox **Select Automatically** again, the system will use the measured scalings instead of the theoretic ones. You will recognize this via the label "**Measured**" instead of "**Theoretic**" beside the pixel size.

3.10 Closing the Software

1. Click on **File** | **Exit**. Alternatively you can use the short cut *ALT+F4* or click on the **Close** icon in the program bar.

Info

If you haven’t saved your files the **Save Documents** dialog will open before the program closes. Select files you want to save or unselect files you don’t want to save.

3.11 Displaying and adapting a grid in the image area

In this section you will find out how to display a grid in your images and how to adapt it.

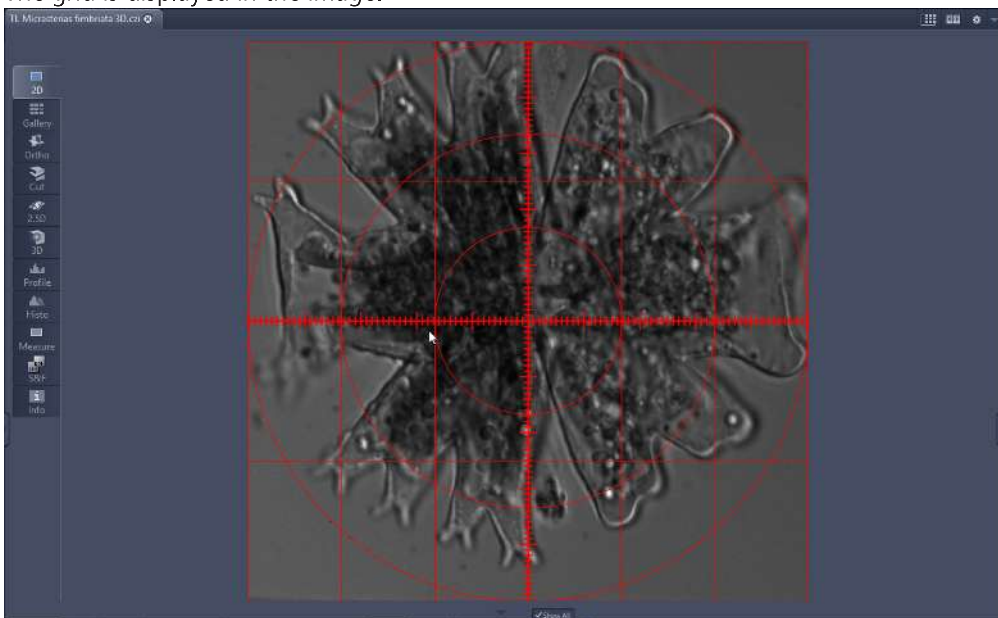
Info

A grid can only be displayed in an acquired image or in a live image in **Continuous** mode.

Prerequisite ✓ You have opened an image in which you want to display a grid.

1. Click on the **Grid** entry on the **Graphics** menu.

   → The grid is displayed in the image.



2. Right-click precisely on a grid line.
   → The shortcut menu opens.
3. Click on the **Format Graphical Elements** entry in the shortcut menu.

→ The **Format Grid** dialog is displayed.

4. Activate the **Synchronize** checkbox. This function means that any changes made, e.g. to the number of columns, are adopted simultaneously for the number of rows. The grid therefore remains square.
5. Set a higher number of columns using the **Columns** slider. Alternatively, you can enter a value in the input field.
   → The changes are displayed directly in the image.
6. Click on the **Save** button to save the grid settings.
   → The Windows dialog for saving settings is opened.
7. Enter a name and click on **Save**.
8. Close the **Format Grid** dialog.

You have inserted a grid into your image, edited the grid and saved your grid settings.

4 Basic Concepts

4.1 Image Acquisition

The software completes all microscopes, microscope systems like LSM, and cameras from ZEISS to efficient and tailor-made imaging systems. With little training you will interactively control the entire workflow from image acquisition, processing and analysis.

Depending on the system you can capture single images, multi-channel fluorescence images or video sequences with up to 16-bit per channel image information. The software contains the so called 'Smart Setup' which automatically delivers several proposals for the optimal dye and wavelength combinations for an experiment.

A wide range of different cameras can be used, starting from simple consumer cameras through to high-resolution and high-sensitivity microscope cameras. The seamless integration of cameras into the software allows you to acquire complex images and image sequences by one mouse click. For best results we recommend to use ZEISS Axiocam microscope cameras.

4.2 Image Processing

After acquiring an image it is immediately displayed on your screen. It can then be optimized using a wide range of techniques:

- Contrast, brightness and color adjustment
- Noise suppression, smoothing and contour enhancement
- Sharpness enhancement/emphasizing of details
- Correction of illumination influences and white balance

The software can also be used to add any annotations that you may require to the images. All elements, from scale bars and colored markings through to text and graphics, have been integrated into the program.

4.3 Image Analysis

Even with **ZEN lite** you are able to perform simple interactive measurements. The measured values (e.g. lengths, areas and perimeters) are made available in a data table, and can be processed further using spreadsheet programs.

With the optional modules **Image Analysis** and **Measurement** you can perform professional analysis tasks like generating automatic measurement procedures or measuring microscopic structures interactively.

4.4 File Format

For the ZEN software we developed a special file format called ***.czi** (Carl Zeiss Image). Besides the image data itself, the image format saves a lot of additional data, for example the date of acquisition, microscope settings, exposure values, size and scale details, contrast procedures which were used. Also all annotations and measured values are saved with the file.

To learn more about the ZEISS image file format we recommend to visit the ZEISS Microscopy Community forum in the internet (http://forums.zeiss.com/microscopy/community/forum.php). There you can join interesting discussions or download the detailed documentation of the file format.

Fig. 7: CZI file format

4.5 Extensions

The extensions concept allows you to extend ZEN dynamically in its functionality. From a technical point of view the concept is comparable with plug-in`s or add-on`s. For the extensions we reserved a special area (*Extensions tab [▸ 666]*) within the software so that you can find all loaded extensions at a glance.

5 User Management

The software can be used with or without user management.

Without user management

User management is disabled by default. This means that every user has the same rights. No username or password is required and there are no user roles within the software (i.e. the user can perform any action).

With user management

If user management is enabled, each user has an account which is used to log into the software. Each user account is assigned to one or more user groups.
User groups define the privileges (actions the user can perform in the software) for the users assigned to the group. Groups typically correspond to the roles in the software (e.g. Administrator, User). However, you can also create new user groups if required.
Typically, one user is assigned to one group, but can be assigned to multiple user groups if required. Users have the sum of all permissions of the groups to which they are assigned.

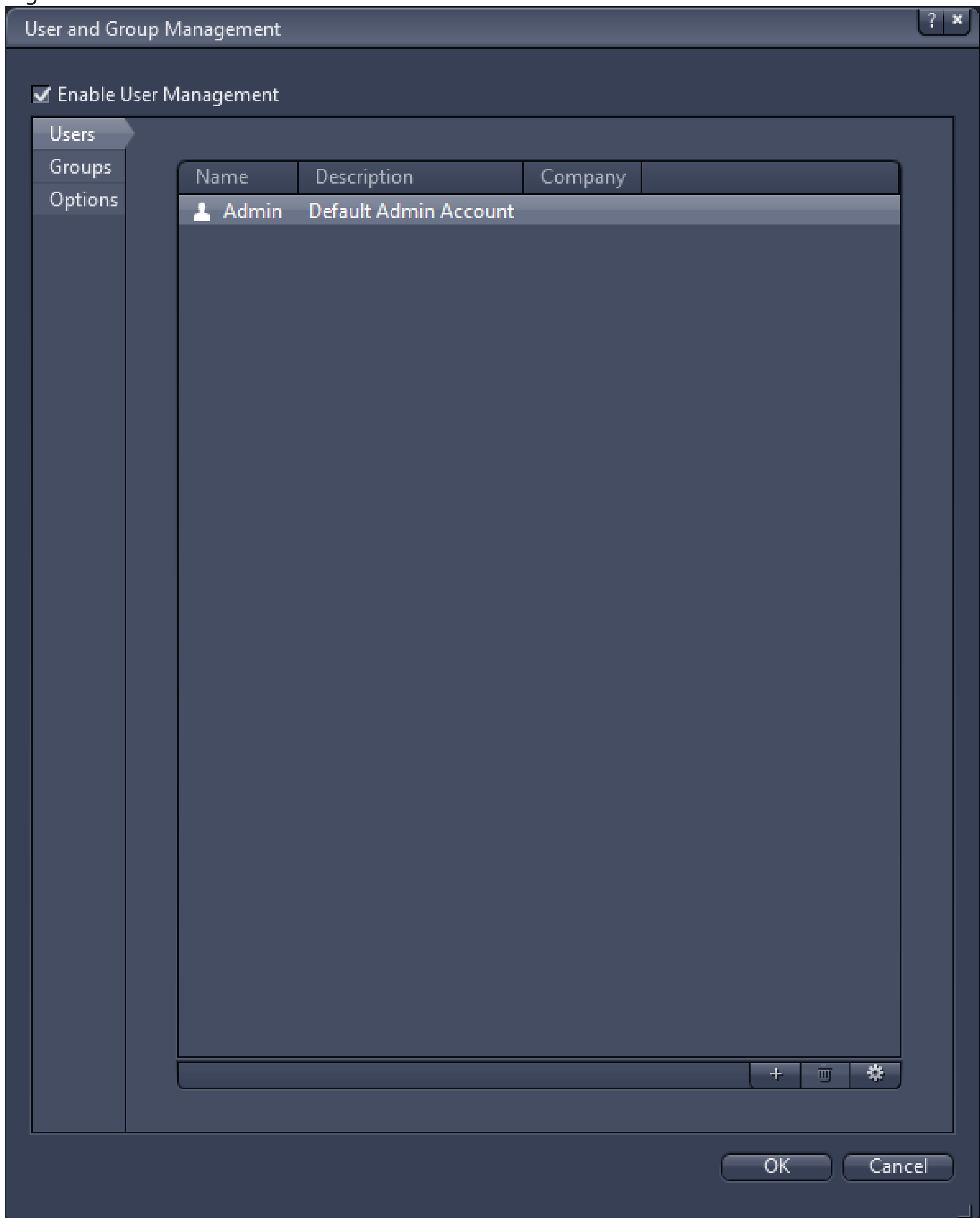
When you start ZEN with user management, you have to enter your username and password on the login screen. Additional to the general login, the last three logged in users on this machine are displayed on the login screen.

ZEN Data Storage

If you use the ZEN Data Storage and user management, the users are the data storage specific ones.

5.1 Creating a new user

1. Click on **Tools > Users and Groups...** to open the **User and Group Management** dialog.



2. Activate **Enable User Management** checkbox.

   → As the user management is enabled now all settings will be effective with the next start of the software. Make sure that you remember password, username, etc.

3. In the **Users** tab, click on the **Add** button [+].

→ The **New User** dialog opens.

4. Enter a **user name**.
   Optional: Enter a **description** and/or enter and confirm a **password**.
5. Click on **OK**.

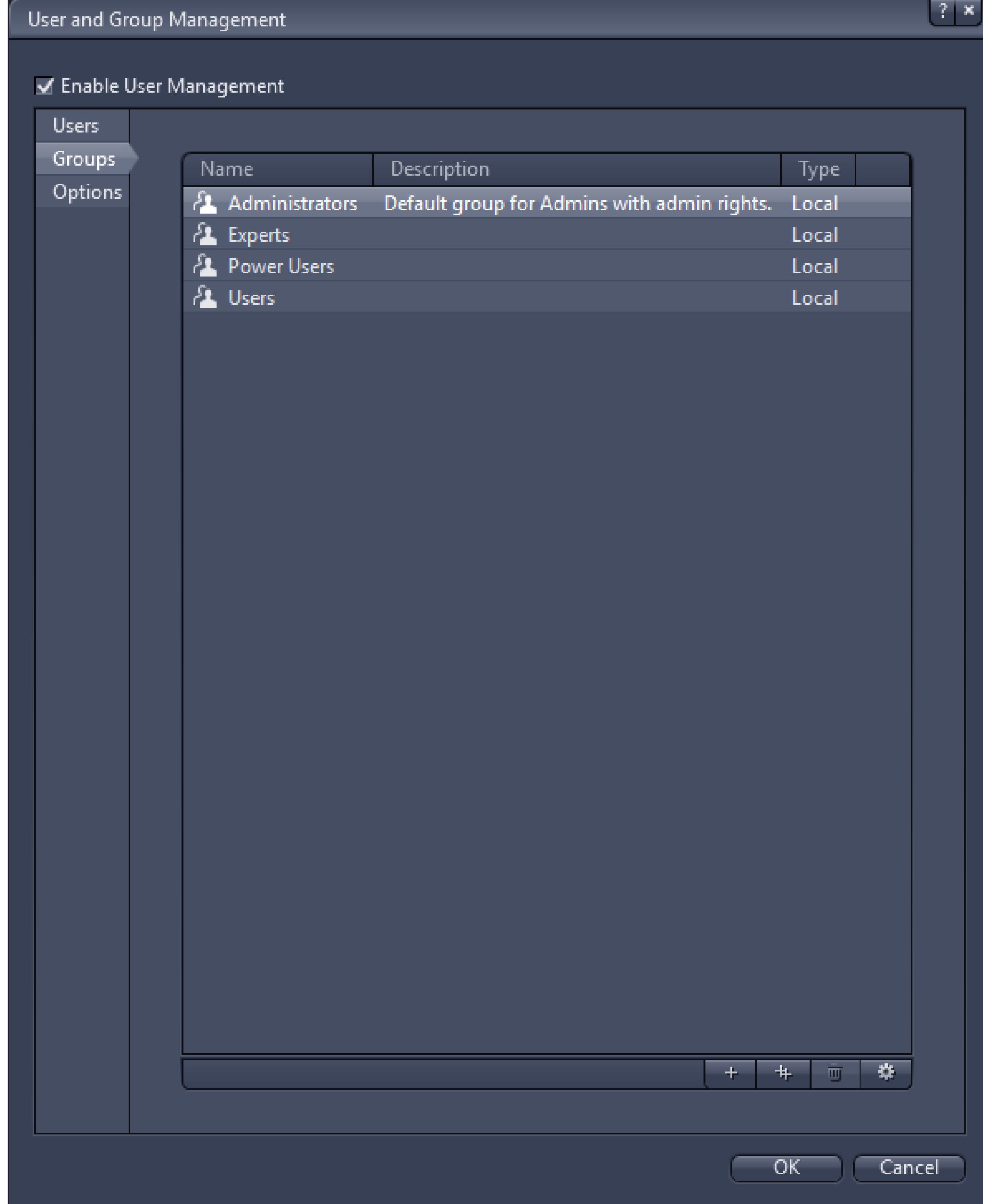
You have successfully created a new user. Now you can now add the user to a specific user group.

5.2 Adding users to a group

Prerequisite

- ✓ You are in the **User And Group Management** dialog (**Tools > Users and Groups...**) .
- ✓ **Enable User Management** is activated.

1. Click on the **Groups** tab.



→ By default you will find 4 groups (**Admin**, **Expert**, **PowerUser**, **User**) with each group having the same rights. Learn how you can manage access rights for user groups under *Managing access rights for user groups [▸ 40]*.

2. Select the group you want to add a user to, e.g. **User**.
3. In the list, click on the **Group Properties** button.
   → The **Group Properties** dialog opens. Under **Data > Members** you see the members list.
4. Click on **Add Member**.
   → The **Select User** dialog opens.
5. Select the user you want to add to the group and click on **OK**.

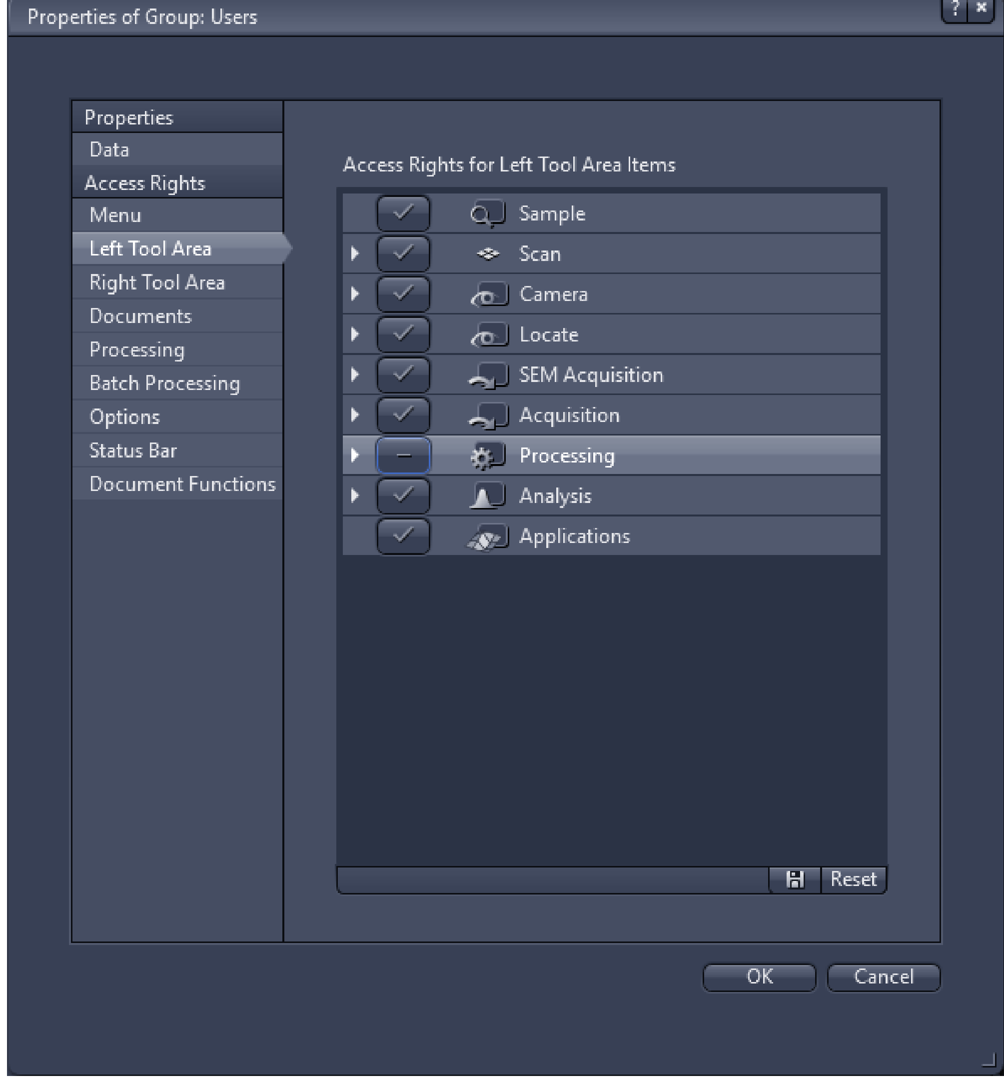
You have successfully added a user to a user group.

5.3 Managing access rights for user groups

You can restrict the access for user groups to certain functionalities of the software. If you use ZEN Data Storage, you can also assign privileges to user groups. For more information on that, see *Managing group privileges [▸ 41]*.

Prerequisite ✓ You are in the **User And Group Management** dialog (**Tools > Users and Groups...**) .

1. Click on the **Groups** tab.
   → By default you will find 4 groups with each group having the same rights.
2. Select the group you want to manage access rights for.
3. Click on the **Group Properties** button .
   → The **Group Properties** dialog opens.
4. Under **Access Rights** click on the **Left Tool Area** tab.
   → You see a list with elements of the **Left Tool Area**.
5. Click on the **Check mark** button in front of the entry **Processing**.
   → The button changes to an **Minus** icon. The access to the **Processing** tab is now restricted for all members of this group.



6. Click on **OK** to close the dialog.
7. Restart the software for the changes to be effective.

You have successfully changed access rights within a user group.

5.4 Managing group privileges

If you use ZEN with ZEN Data Storage, privileges are assigned to user groups. They specify what actions members of the group can perform in the software.
The software contains various pre-defined roles, each with different sets of privileges. Typically, the software contains one user group for each role. However, you can create any number of user groups with arbitrary privileges.

Prerequisite
- ✓ You are logged in as an administrator.
- ✓ **Enable User Management** is activated.

1. Got to **Tools > Users and Groups.** .
2. Click on **Groups**.
3. Select the desired user group and click on .
4. Click on **Privileges**.
   → The privileges for the ZEN Data Storage groups are displayed. Each privilege is displayed with its **Name**, a **Description**, and the **Application Name**. Here you can see which privilege is designated for groups in **ZEN**, **ZEN core**, or the **ZEN Storage Processing Server**. If the field **Application Name** is empty, the respective privilege is generally available.
5. Select the privileges for the user group.
   → You can click on one of the pre-defined **Privilege sets** or activate individual checkboxes to create a custom set of privileges.
6. Click on **OK**.

You have now set/changed the privileges for this group.

5.5 Options

The options apply to all users, regardless of the user groups to which the user is assigned. If you are using ZEN Data Storage, not all parameters are displayed.

Parameter	Description
Check the following rules for a password	Here you can specify certain rules or criteria for a password that is created. If the checkbox is activated, the rules must be fulfilled when a new password is created. The following rules can be adjusted: ▪ Minimal number of lower case characters (e.g. when you set "2", the password must contain at least two lower case characters e.g. "e" and "f") ▪ Minimal number of upper case characters (e.g. when you set "2", the password must contain at least two upper case characters e.g. "C" and "G") ▪ Minimal number of digit characters (e.g. when you set "3", the password must contain at least 3 digits (from 0 - 9), e.g. "5", "6", "7") ▪ Minimal number of special characters (e.g. when you set "1", the password must contain at least one special character, e.g. "&") ▪ Minimum length (e.g. when you enter "9", the password must consist of at least 9 characters (any from above).

Parameter	Description
Do not allow Windows or ZEN user name as password	If activated, it is not allowed to use an existing user name from Windows or ZEN installation as password for the software.
Disable the reuse of last used passwords	If activated, you can enter a certain number of passwords which can`t be reused after each other. E.g. if you enter the number '**3**' you have to assign 3 different passwords one after another before you can use (reuse) an old password.
Disable the use of common passwords	If activated, you can create and edit a list which contains passwords which you can lock for usage. E.g. if you add the entry '123456789Password' this password can not be assigned from a user.
Force users to change password after period of time	**Activated**: The user must change his password after the specified period of time elapses. **Deactivated**: The password never expires.
– Days before expiry	Specifies the period of time after which the password expires.
Lock user after wrong password entries	If activated, you can determine the number of attempts the user has if he enters a wrong password. E.g. if you enter '**3**' , the user can enter a wrong password for 3 times before his user account is locked.
Lock screen after certain time span	**Activated**: After a period of inactivity the screen is locked and the user must enter his/her password to continue working. **Deactivated**: The password never expires.
– Minutes until screen lock	Specifies the time span after which the screen is locked.
Enable Auto-Login	▪ Activated: No password is required The user is logged in automatically based on the Windows username. Create a user group in the software that is based on Windows Active Directory (**Type = AD**) and ensure that all relevant Windows users are present in the group and that the group has sufficient privileges in the software. ▪ Deactivated: Each user has to log in with their own password.
Export/Import user database	Enables you to export or import the user database, including all user groups and privilege sets, for example to exchange it with another system.
– Export...	Specify the location on the file system where the database should be exported
– Import...	Select the database location on the file system

6 Image Acquisition (General)

6.1 Acquiring Multi-Channel Images with Cameras

In the following chapters you will learn how to set-up and run multi-channel experiments quick and easy.

Info

Make sure that you work with a fully motorized microscope system. In advance all microscope components (e.g. objectives, filters, etc.) must be configured correctly in the MicroToolBox (MTB) software.

In principle there are two variants for setting up multi-channel experiments. The first variant uses **Smart Setup**, while the second variant uses the **Channels** tool. Both variants have similarities and differences, which are presented in the following overview:

Commonalities

- Fluorescent dyes and transmitted light techniques can be selected from a database.
- Hardware settings for motorized microscopes, which take the properties of the selected dye and the available microscope hardware into account, can be created automatically.
- Bases for experiments can be created using both variants and experienced users can optimize settings further.

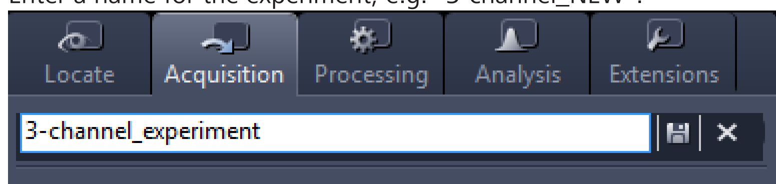
Differences

Smart Setup	Channels tool
A maximum of 4 camera channels, 8 confocal channels and 1 transmitted light channel are available	No restriction on the number and type of channels
Offers up to 3 proposals of variants of the experiment (depending on the selected combination of dyes and available hardware)	-
Offers more optimization of experiment settings by using the Motif buttons	-
Graphic overview of the expected signal strength for the selected dyes	-
Graphic overview of the expected spectral crosstalk with the selected dye combinations	-
Display of the excitation and emission spectra of the selected dyes	-
-	Channels can be configured for dyes that are not supported (or not supported sufficiently well) by the available hardware

6.1.1 Set up a new experiment

Prerequisite
- ✓ You have switched on and configured your microscope system and all components.
- ✓ You have successfully started the software.

1. In the **Left Tool Area** click on the **Acquisition** tab.
2. In the **Experiment Manager** click on the **Options** button.
   → The **Options** dropdown list opens.
3. To create a new, "empty" experiment, click on the **New** entry.
4. Enter a name for the experiment, e.g. "3-channel_NEW".



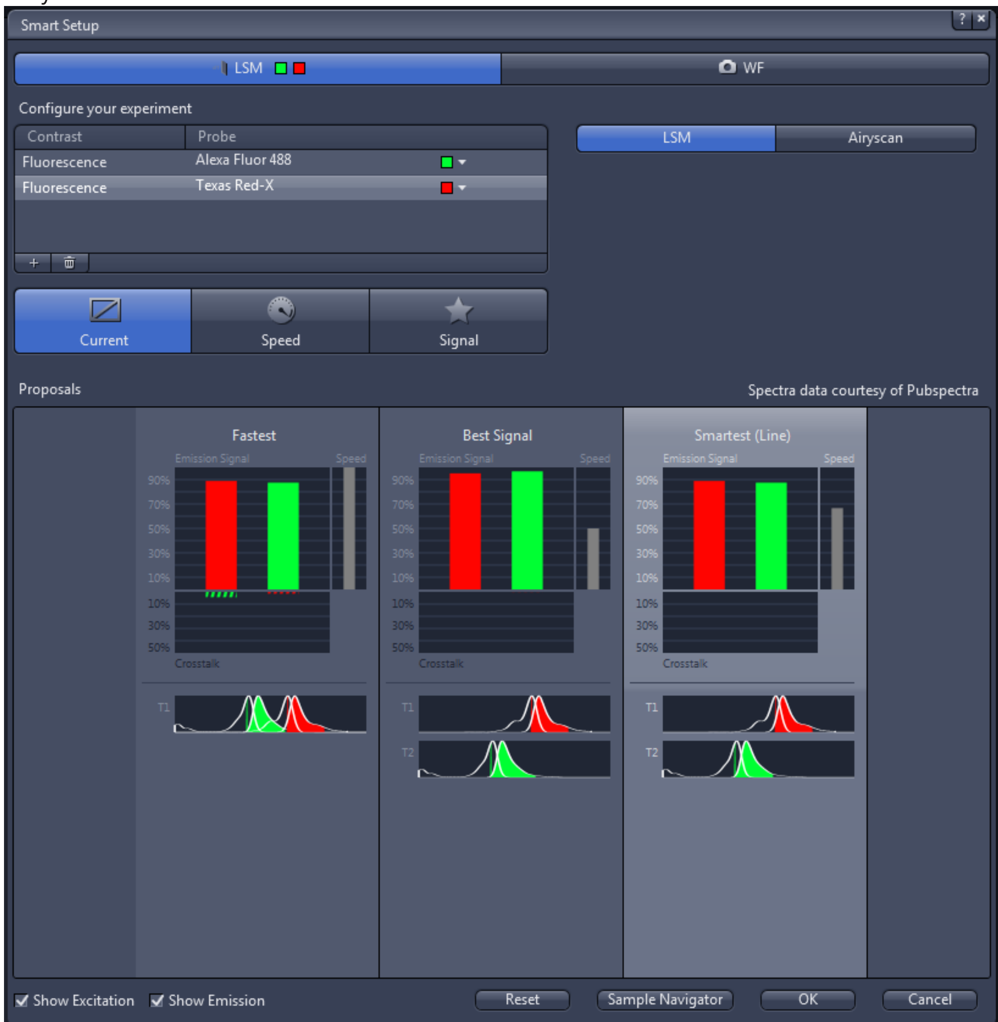
5. To create the experiment, click on the **Save** button.

You have created a new, blank experiment. All other settings are now stored in this experiment. If you make changes to the experiment, an asterisk (*****) after the file name appears. This means that the experiment was modified and not saved. Save your experiments from time to time to ensure that your settings are not lost.
Note that with a very high number of experiments saved in the Experiment Manager (more than 500) moving from the **Locate** to the **Acquisition** tab takes a considerably long time. Delete redundant or no longer used experiments to avoid this lag time.

6.1.2 Variant 1: Smart Setup

1. Click on the **Smart Setup** button on the **Acquisition** tab.
   → The **Smart Setup** dialog opens.
2. Select the **WF** button on top of the dialog.
3. To add a channel, click on the **Add** button in the **Configure your Experiment** section.
   → The **Add Dye or Contrasting Method** dialog opens.
4. Select the desired dye or contrast method.
5. Click on the **Add** button. Alternatively you can double-click on the entry in the dye database. The dye is then adopted directly into the experiment.
   → You have added a channel to your experiment. To add further channels, repeat the last 2 steps.
6. To return to **Smart Setup**, click on the **Close** button.

→ You will now see a graphic overview in the **Proposals** section. This displays the spectra of the dyes, the expected signal strengths per dye and the spectral crosstalk schematically.



7. To select a proposal (if there's more than one), for all active configured channels, activate the proposal.
8. To optimize experiment settings additionally, click on a **Motif** button.
   The **Automatic** button is set as default.
9. To optimize experiment settings, adopt the suggestion and leave **Smart Setup**, click on the **OK** button.
   → The added channels are adopted automatically into the **Channels** tool.
10. Click on the **Set Exposure** button in the **Action buttons** bar on top of the **Acquisition** tab.
    → The exposure time is now measured for all three channels one after the other. This is adopted into the settings for the channels. Following the measurement of the exposure time, the multi-channel image is acquired automatically and displayed in the **Center Screen Area**.
11. To save the experiment together with all the settings, click in the **Experiment Manager** on the **Options** button .
12. In the **Experiment Manager** click on the **Save** entry in the dropdown list.

You have set up the multichannel experiment using **Smart Setup**, executed it and then saved the configuration. This means that you can repeat the experiment as often as you like using the same settings.

6.1.3 Variant 2: Channels Tool

Info

This workflow does not work with LSM systems.

1. Open the **Channels** tool in the **Acquisition Parameter** group.

2. From the drop down list, select the channel.
   → The **Add Dye or Contrast Method** dialog opens.
3. Select the desired dye or contrast method. You can search for a dye by entering its name (or starting letter) in the **Search** input field.
4. Click on the **Add** button at the bottom of the dialog or simply double click on an entry.
   → The channel will be added to your experiment. To add more channels, repeat the last 2 steps.
5. Click on the **Close** button.
   → You will see the added channels in the **Channels** tool.
6. Click on the **Set Exposure** button in the main buttons bar on top of the **Acquisition** tab.
   → The exposure time is now measured for all active configured channels one after the other. It will be also adopted into the settings for the channels. To set the exposure time channel specific, use the **Set Exposure** button in the channel settings of the channels tool.
7. To save the experiment together with all the settings, click in the **Experiment Manager** on the **Options** button.
8. Click on the **Save** entry in the dropdown list.

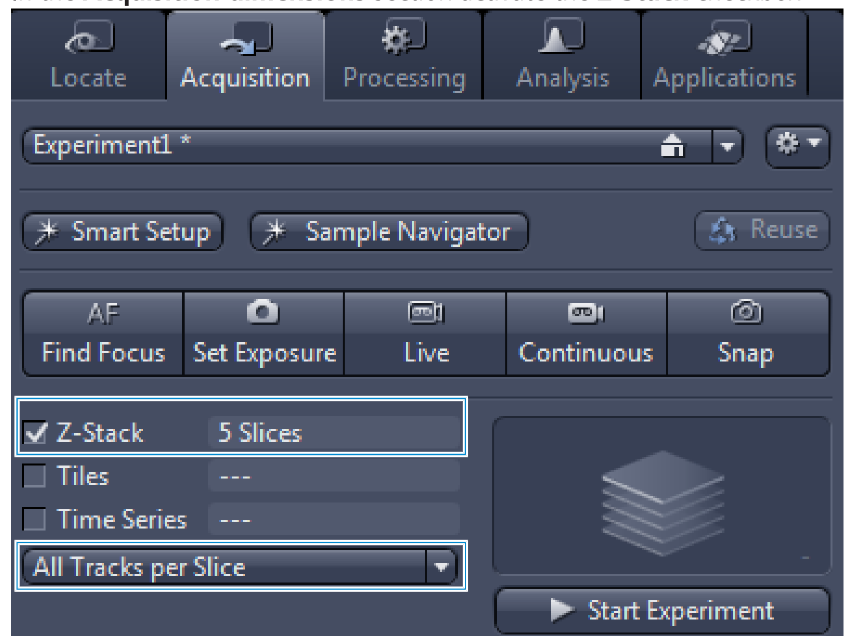
You have set up the multichannel experiment using the **Channels** tool, executed it and then saved the configuration. This means that you can repeat the experiment as often as you like using the same settings.

6.2 Acquiring Z-Stack Images

Prerequisite

- ✓ You have licensed **ZEN celldiscoverer**, **ZEN slidescan**, **ZEN system**, or **ZEN pro** and additionally licensed the **Z-Stack** module.
  For LSM systems, the **Z-Stack** module is part of the system license.
- ✓ You have switched on and configured your microscope system including all components.
- ✓ You have *set up a new experiment* [▸ 44], at least defined one channel and adjusted focus and exposure time correctly.
- ✓ You are on the **Acquisition** tab.

1. In the **Acquisition dimensions** section activate the **Z-Stack** checkbox.



→ The **All Tracks per Slice** selection box appears below the check boxes.

→ The **Z-Stack** tool appears in the **Multidimensional Acquisition** tool group.

2. Use the **All Tracks per Slice** option or select the **Full Z-Stack per Track** option in the selection box.
3. Simply click on the blue bar to open the **Z-Stack** tool.

You have successfully completed the general preparations. You can now set up Z-Stack experiments automatically or manually.

6.2.1 Configuring a Z-Stack automatically

Info

Note that the automatic Z-Stack configuration will only work, if no LSM tracks are added in the **Channels** tool.

1. Make sure that you have placed a sample in the visual field of the camera and that the sample is roughly in focus. Set the exposure time of the camera fair enough for receiving a good signal.
2. On the **Acquisition** tab, in the **Z-Stack** tool, click on **Start Auto Configuration**.
3. Confirm the system message by clicking on **OK**.

   → The automatic configuration starts.

   → The auto configuration sets the focus position for the first, last and center slice of the Z-Stack, the number of slices and the interval automatically. The Z-Stack experiment is set up successfully now.
4. Click on the **Start Experiment** button to start the experiment.

You have successfully set up and performed an z-stack experiment.

Info

- The auto configuration can last for a few seconds up to half a minute depending on the acquisition settings. You can check the configuration status on **Progress bar** in the **Status bar**.
- You can change the area of the sample (Z direction in %) covered by the Z-Stack auto configuration under **Tools > Options > Acquisition > Z-Stack** section. Smaller values will enlarge the Z-Stack. Bigger values will make the Z-Stacks smaller.

6.2.2 Configuring a Z-Stack manually (First/Last Mode)

Using this mode you set the first and the last plane of the Z-Stack. This mode is suitable if you don’t know the thickness of your sample exactly.

Prerequisite ✓ You are in the **Z-Stack tool**.

1. Activate the **First /Last** mode by clicking on the **First /Last** button. This button is selected by default.
2. In the **Live** mode adjust the Z-drive until you have reached the upper plane of the Z-Stack. The blue plane in the illustration shows the actual focus plane.
3. Click on **Set First** button to set the adjusted Z-Position as first position of the Z-Stack.
4. In the **Live** mode adjust the Z-drive until you have reached the lower plane of the Z-Stack.
5. Click on **Set Last** button to set the adjusted Z-Position as last position of the Z-Stack.
   → You have set the upper and lower boundaries of the Z-Stack.
6. Click on the **Optimal** button. This will adjust the number of slices and the best interval according to the Nyquist criteria. Alternatively you can set the desired interval and number of slices in the input fields manually.
7. Click on **Start Experiment** button to start the experiment.

You have successfully set up and performed an Z-Stack experiment using **First/Last** mode.

6.2.3 Configuring a Z-Stack manually (Center Mode)

Using this mode you set the center plane of the Z-Stack. This mode is suitable if you know the thickness of your sample. It will be the fastest method to set up a Z-Stack in this case.

Prerequisite ✓ You are in the **Z-Stack tool**.

1. Activate the **Center** mode by clicking on the **Center** button on the top of the tool.
2. In the **Live** mode adjust the Z-drive until you have focused the center of the sample exactly. The blue plane in the illustration shows the actual focus plane.
3. Click on the **Center** button under the settings section to set the actual focus position as center of the Z-Stack.
4. Click on the **Optimal** button. This will adjust the number of slices and the best interval according to the Nyquist criteria. Alternatively you can set the desired interval and number of slices in the input fields manually.
   → Depending on which option is selected in the **Keep** section either the **Interval** or the **Slices** will be held constant.
5. Click on **Start Experiment** button to start the experiment.

You have successfully set up and performed a Z-Stack experiment using the **Center** mode.

6.3 Acquiring Time Series Images

Prerequisite

- ✓ To set up **Time Series** experiments you need to license the **Time Series** module. For LSM systems, the **Time Series** module is part of the system license.
- ✓ You have set up a *new experiment [▸ 44]*, at least *defined one channel [▸ 43]* and adjusted focus and exposure time correctly.
- ✓ You are on **Acquisition** tab.

1. Activate the **Time Series** tool by activating the **Time Series** checkbox in the **Acquisition Dimensions** section.
   → The **Time Series** tool appears in the **Left Tool Area**.
2. Open the **Time Series** tool.
3. Set length of your time series by the **Duration** slider. You are able to select an interval (days, hours, minutes, seconds, milliseconds) or the cycles (1-n) e.g. 10 cycles.
4. Set interval of your time series by the **Interval** slider, e.g. 5 s.
5. Click on **Start Experiment** button.

The time series experiment will be started. You`ve successfully learned the basics of how to set up time series experiments. In our example in 10 cycles after each 5 seconds an image is acquired. The Time Series image also contains 10 single images.

Info

You can display the individual images via the **Time** slider on the **Dimensions** tab.

6.4 Acquiring a Panorama Image Automatically

The field of view of your microscope might be too small for the sample area you wish to acquire. You can automatically visualize panorama images from a sample area which is larger than the camera sensor can cover by means of a single snap.

With the **Live Panorama** tool, you can move the stage while the software automatically acquires individual images, stitches them together and creates a panorama image.

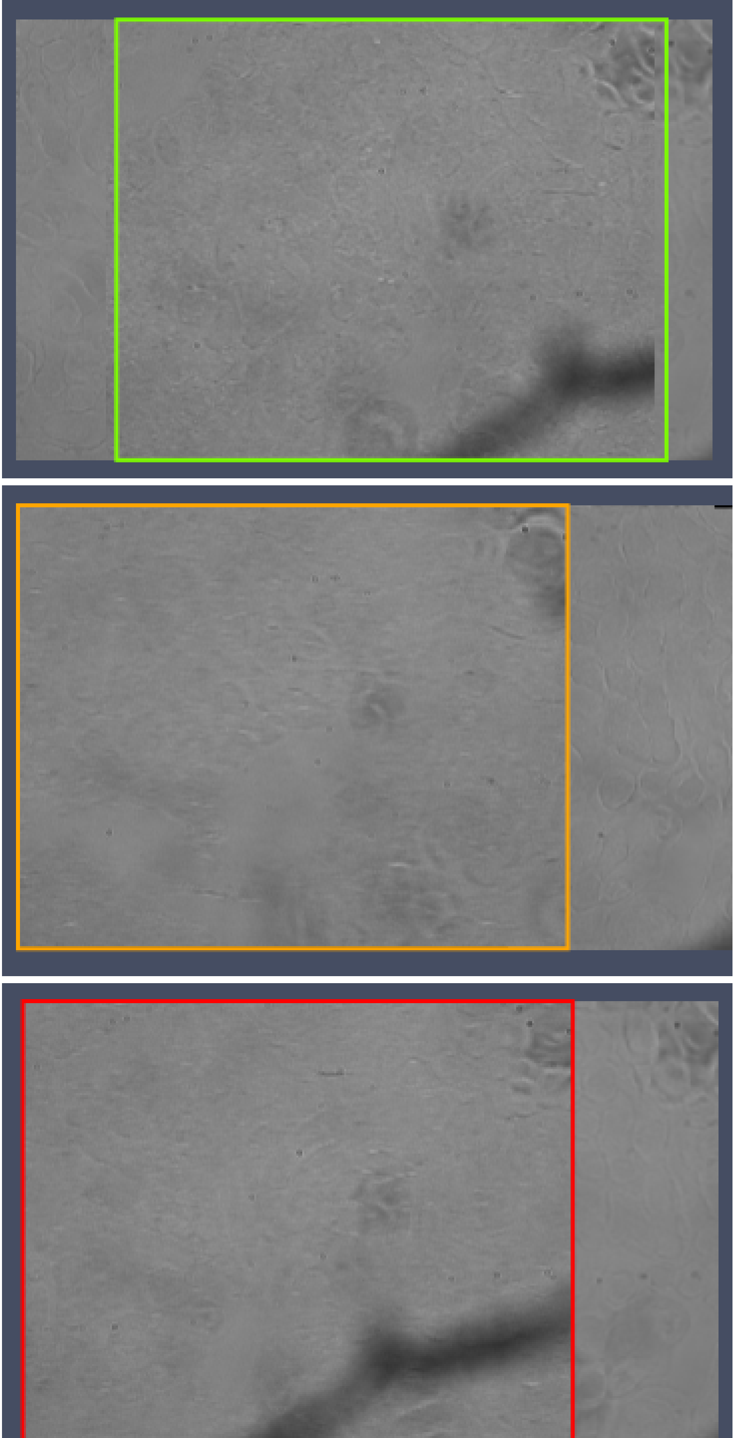
Note that **Live Panorama** works for uncoded and un-motorized stages as well as motorized stages.

Prerequisite

- ✓ You have set-up and configured your microscope system correctly.
- ✓ You work with brightfield or widefield illumination.
- ✓ Your image has sufficient contrast. Lower magnificantion objectives typically give better results.
- ✓ A sample is on the stage and stays in focus. Note that you can adjust the focus during the Live panorama.
- ✓ You have started the software and selected the **Locate** tab. If you work with an LSM, activate camera mode.

1. On the **Locate** Tab, click the **Live** button to get a live image from the microscope camera. Adjust the camera and microscope settings to see a well illuminated and sharp live image.
2. Navigate to a specific area on your sample you want to image.
   → Move the stage gently and not too fast!
3. Select the **Live Panorama** tool, and click the **Start Live Panorama** button.
   → After a short moment, the camera rectangle changes to green and you can move the stage. The panorama acquisition starts. You see the live image of the sensor area.

→ Note that the color of the rectangle changes to orange or red, when the software loses the stitching algorithm. This might happen if the stage is moved too quickly. Then you have to manually go back to the last known or successfully synchronized position. If you wish to image a continous area of the sample without any gaps, we recommend using a zig-zag pattern to move over your sample to slowly build up the image.



4. Move the stage slowly in the desired direction.
   → During the stage movement the software automatically acquires the panorama image.
5. To finish the acquisition, click **Live Panorama** tool **| Stop** button.
   → A message is displayed, prompting you to decide if you want to generate an image pyramid to optimize the later display of your image.
6. Click **Yes**.

The panorama image is added to the **Documents** tab **|** **Images and Documents** tool.

Stitching artifacts can be corrected by making sure the camera rotation has be corrected. You can make use of the stitching image processing function to correct them.

You can save the image to your file system.

For more information, see *Live Panorama tool [▸ 683]*.

6.5 Acquiring EDF Images Manually (ZEN lite)

The module **Manual Extended Focus** for **ZEN lite** allows to create one single image, out of several acquisitions with different focus positions. The sharp areas of all acquisitions are combined to one consistently sharp image, the so called EDF image (EDF = Extended Depth of Focus).

Preconditions

To work with the module **Manual Extended Depth of Focus**, the following conditions must be fulfilled.

- You have licensed your module and ensured that in the menu **Tools > Modules Manager** the function **Manual Extended Focus** is activated via the checkbox.
- The tool **Manual Extended Depth of Focus** is now shown on the **Locate** tab.

6.5.1 Prerequisites

Prerequisite ✓ You are on the **Locate** tab.

1. Click on the **Live** button to open the live image.
   → The **Live** button changes to a **Stop** button during a live image.
2. For the extended focus acquisition look for a suitable position on the sample.
3. Click on the **Stop** button to switch off the live image.

You have made all necessary arrangements. For EDF image acquisition you have now three modes available. These will be revealed in the following chapters.

6.5.2 Acquiring EDF image with Timer mode

In this mode, after a freely selectable interval, a new picture will be automatically added. In the period between the acquisitions you can set a different focus position. After each recording a new image with an extended depth of focus is immediately calculated. When you finish the acquisition you will get the image which was calculated last.

Prerequisite ✓ You are in the tool **Manual Extended Depth of Focus.**

1. To acquire Z-Stacks, activate **Z-Stack**.
2. Select the **Timer** mode.
3. Adjust the length of your interval by the **Interval** slider. Set the interval long enough, to move the specimen comfortably to a new focus position. After lapse of time, an image of your actual position will be automatically acquired.
4. Click on the **Start** button to start the acquisition. The **Start** button will only be active when the live image is closed.
   → The **Central Screen Area** will be split into two parts. Right you will always see the current live image, left you will see after each new acquisition the actual calculated extended focus image.

5. Set a new focus position after each acquisition. Repeat this procedure until you are satisfied with the result.
6. To finish your acquisition, click on the **Stop** button. If you click on the **Pause** button you are able to pause the acquisition.

You have successfully acquired an image, showing the extended depth of focus, with the **Timer** mode.

6.5.3 Acquiring EDF image with F12 Key mode

In this mode you can achieve different time intervals between the individual focus steps. By pressing the *F12* key on your keyboard, you can manually record a new image. Between the acquisitions, you can change the focus position.

Prerequisite ✓ You are in the tool **Extended Depth of Focus.**

1. To acquire Z-Stacks, activate **Z-Stack**.
2. For **Mode**, click on **F12 Key**.
3. Click on the **Start** button. The **Start** button will only be active when the live image is closed.
4. Press the *F12* key on your keyboard to initiate manually, each acquisition with a different time step.
5. Select manually a new focus position.
   → Repeat the last two steps until you have sharp images from all desired areas of your sample.
6. To finish the acquisition, click on the **Stop** button.

You have successfully acquired an image, showing the extended focus, with the *F12* key mode.

6.5.4 Compare Images using Split Display

Prerequisite ✓ You have already acquired images with extended depth of focus which you want compare to each other.

1. To compare images click on the **Create new multiple image** button .
2. In the **view options** on the **Split Display** tab define an arrangement of 2x2 images to be shown aside .
3. Move the images via Drag & Drop from the **Image and Documents** gallery in the right tool area, to the **Center Screen Area**. If necessary, click the **show/hide** button to show the **Image and Documents** gallery.

The comparison of all 4 methods used (via Timer, F12-key, Z-stack without shift, Z-stack with shift) shows identical resulting images.

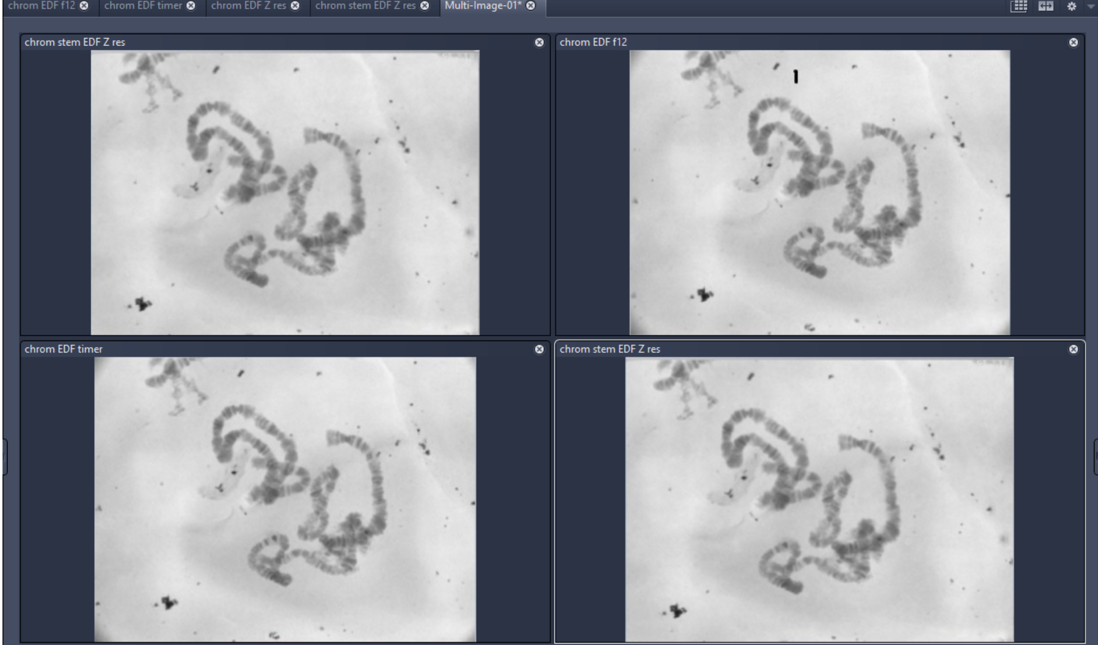


Fig. 8: Image comparison with multi image

6.6 Cryo Acquisition in ZEN

To be able to acquire cryo images in ZEN, several prerequisites have to be fulfilled:

- The Linkam Cryo stage has to be set in the MTB configuration.
- You have licensed the **Linkam Cryo** module.
- In **Tools > Modules Manager > Optional Hardware**, the **Linkam Cryo** module has to be activated.
- To be able to control the hardware, you need the software version **ZEN Pro** or **ZEN Systems**. All other versions of ZEN can only read the temperatures from image metadata.

6.6.1 Exporting temperature data

You can export the temperature data which are logged during the acquisition of cryo images in ZEN. The export depends on the software version you have started.

Export with ZEN Pro or ZEN System

This export is only possible with ZEN Pro or ZEN System.

Prerequisite ✓ You have opened the image where you want to export the temperature data.

1. In the **Cryo Temperature** tool, click on **Export Temperature Data**.
   → The temperature data is displayed as table in ZEN.
2. In the **Export** tab, click on **Export Table**.
3. In the file browser, select the storage location and click on **Save**.

You have exported the image temperature data.

Export without ZEN Pro and ZEN System

This export is possible in all ZEN versions (also in ZEN Pro or ZEN System).

Prerequisite ✓ You have opened the image where you want to export the temperature data.

1. Click on the **Measure** view.
2. In the Measurement tab, click on **Export Temperature Data**.
   → The temperature data is displayed as table in ZEN.
3. In the **Export** tab, click on **Export Table**.
4. In the file browser, select the storage location and click on **Save**.

You have exported the image temperature data.

6.6.2 Adding temperature annotations to an image

Temperature data of images is saved in the metadata. To display the information directly in the image you need to create an annotation which can be displayed in the image.

1. Open your image in ZEN.
2. In the view options below the image, click on the **Custom Graphics** tab.
3. Click on **Customize**.
   → The **Customize Tools** dialog opens.
4. Under **Frequent Annotations**, select **Other** and click on .
   → The annotation is now visible in the **User Toolbar** list on the top of the dialog.
5. In the **User Toolbar** list, right click on **Other...** and select **Format Custom Graphics**.

→ The **Format new custom graphic tool dialog** opens.

6. In the **Annotation** dropdown list, select **...**.

   → The **Select Metadata** dialog opens.

7. In the list, select your metadata (Cryo Stage Bridge Temperature, Cryo Stage Dewar Temperature, Cryo Stage Chamber Temperatur).
8. Click on **OK** to close the **Select Metadata** dialog.
9. If necessary, change other parameters of the graphic.
10. Click on **OK**.
11. Click on **Close** to exit the **Customize Tools** dialog.

Your custom graphic is now available on the **Custom Graphics** tab. You can add it to your image and see the temperature information displayed directly in the image.

6.7 Using the Dye Editor

The ZEN software is delivered with a large number of preset dyes. Dyes and its parameter are stored in the dye database. In case you have created a custom made filter cube, you may need additional dyes.
You can create new dyes and a custom dye database with the **Dye Editor** in the **Tools** menu.

6.7.1 Dye Editor Dialog

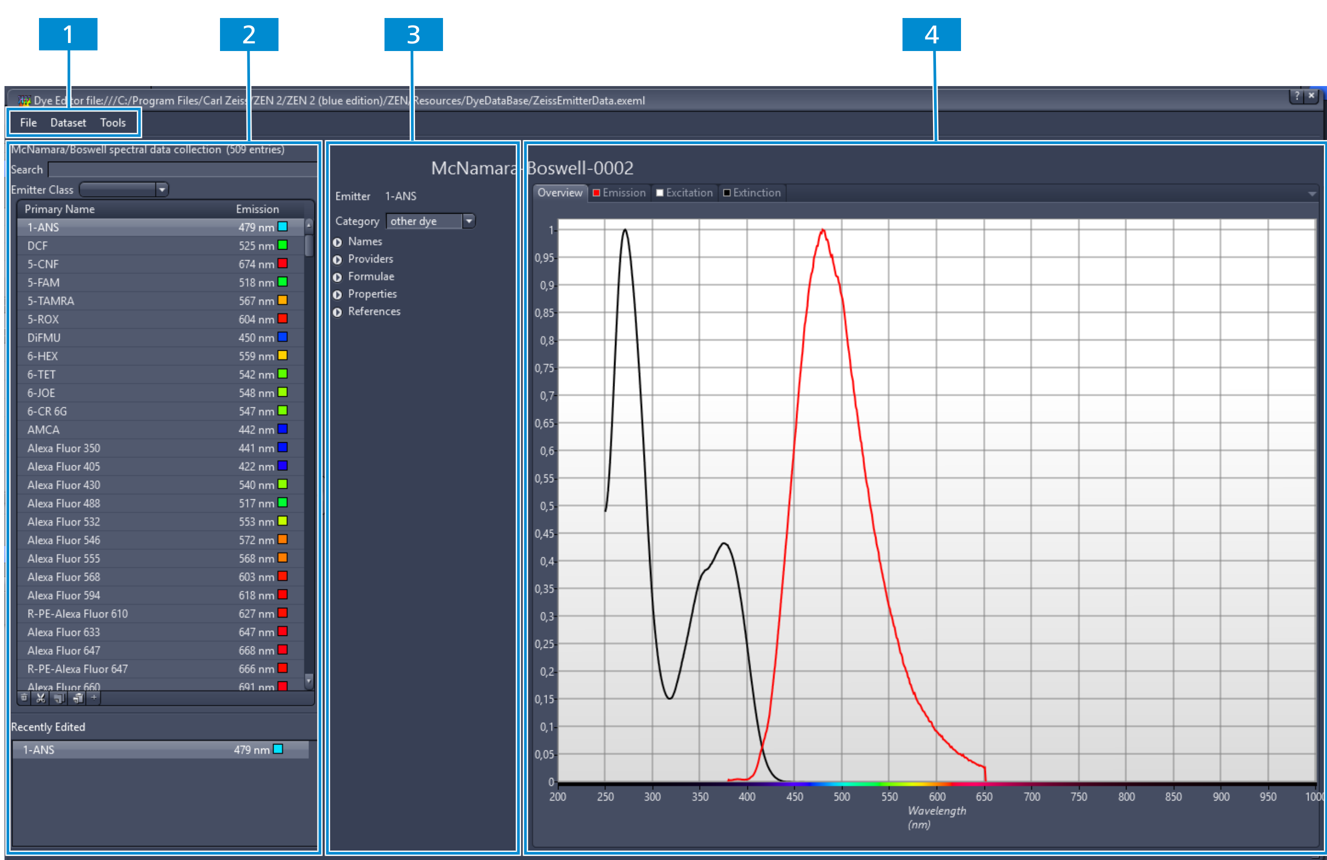


Fig. 9: Dye Editor Dialog

1 **Menu**
Here you can create new data sets and import available dye databases. A detailed description can be found under *Dye Editor Menus [▸ 56]*.

2 **Dye search & database**
In this list you see the dyes that are available in the open database. You can search for dyes and see which dyes you edited last.

3 **Dye information area**
Here you see all the additional information about a selected dye.

4 **Dye spectra**
Here you see the available dye spectra. Click on the relevant tabs to display the **emission**, **excitation**, or **extinction** spectra. On the **Overview** tab you can see all spectra at a glance.

See also

Creating a Custom Dye [▸ 57]

6.7.1.1 Dye Editor Menus

File menu

Menu item	Description	Short cut
New	Creates a new ExEml file in which you can create dye data sets.	
Open file...	Opens a single ExEml file.	
Open folder...	Opens several ExEml files that have been saved together in the same folder.	*Ctrl+S*
Import file...	Imports an ExEml file.	
Save...	Saves the open ExEml file.	
Save As...	Saves the open ExEml file under a new name.	
Save As Folder...	Saves all open ExEml files in a folder.	
Close	Closes the Dye Editor.	*Ctrl+F4*

Dataset menu

Menu item	Description	Short cut
Add new...	Creates a new, empty data set.	
Copy	Copies the selected data set to the clipboard.	*Ctrl+C, Ctrl+Ins*
Cut	Cuts the selected data set and copies it to the clipboard.	*Ctrl+X, Shift+Del*
Delete	Deletes the selected data set.	
Paste	Pastes a data set from the clipboard.	*Ctrl+V, Shift+Ins*
Paste Part	Pastes a range of a data set from the clipboard.	

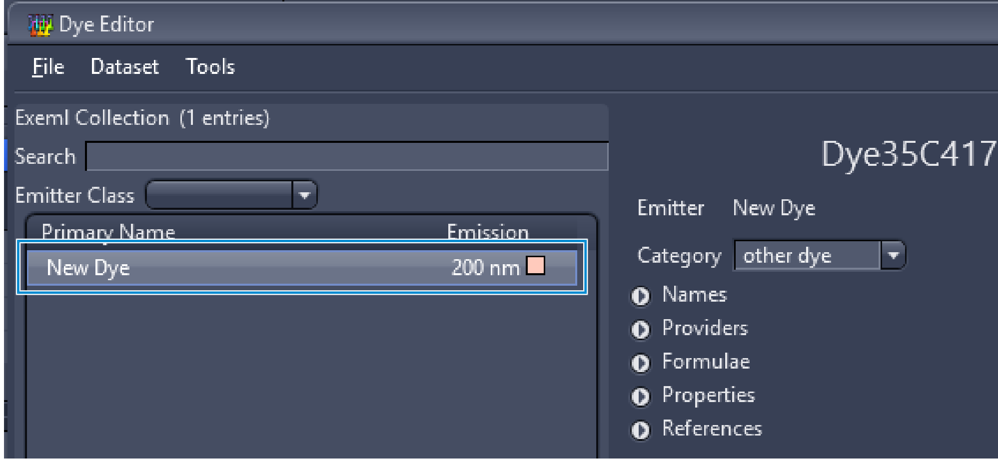
Tools menu

Menu item	Description
Generate short names...	Creates abbreviations for names of dyes. This helps to avoid duplication.
Update max. wavelength emitter properties	Calculates the main emission wavelength of the selected dye from spectral data that has either been copied or entered manually.

6.7.2 Creating a Custom Dye

If non of the preset dyes matches your requirements, you can create a new custom dye.

1. Open the **Dye Editor** via **Tools > Dye Editor...**.
   → The *Dye Editor dialog [▸ 55]* opens.
2. In the **File** menu, click the **Save As Folder...** entry.
3. In the Windows dialog, create a new folder to save your custom dye database, e.g. C:/User/Documents/Carl Zeiss/ZEN/Documents/**New Folder**.
   Name the new folder, e.g. New Dyes.
   → You have created a custom dye database to which all new dyes are stored.
4. Click on the **New Dye** entry in the **Exeml Collection** list.
   → The properties section opens.

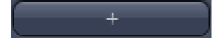


5. In the **Names** section, click in the **Name** input field to change the name of the custom dye.
   → The name enables you to find your custom dye in the **Smart Setup** or **Channels** tool.
6. Fill in other known parameters in the following sections, e.g. **Providers**, **Properties**, **References**.
7. Change to the **Emission** tab.
8. To fill in **Wavelength (nm)** and **Spectrum value** to the table on the right, you have 3 options:

- *Type in data manually. [▸ 58]*
- *Copy and paste data from e.g. an Excel sheet. [▸ 58]*
- *Copy and paste data from a preset dye. [▸ 58]*

6.7.2.1 Type in Data Manually

Prerequisite
- ✓ You have created a new dye in the **Dye Editor** dialog.
- ✓ Your are on the **Emission** tab.

1. In the **Wavelenth (nm) | Spectrum value** table, click on the **Plus** icon.
   Add 10 rows, by pressing *Shift* and the **Plus** icon.
   Add 100 rows, by pressing *Alt+Shift* and the **Plus** icon.
   
2. Type in the corresponding **Spectrum Value** in the input fields.
   → The emission graph is displayed next to table.
3. Save the dye via **File** | **Save...**.

You have saved a new dye in your custom database.

You can use the new dye in the **Smart Setup** or in the **Channels** tool.

6.7.2.2 Copy Data from an Other Source

If you have recorded **Wavelength (nm)** and **Spectrum value** of your custom dye in an other source e.g. an Excel sheet, you can copy the data and paste it directly into the **Dye Editor** dialog.

Prerequisite
- ✓ You have created a new dye in the **Dye Editor** dialog.
- ✓ You have opened your source, e.g. an Excel sheet with the emission data.

1. Arrange the data in two columns like on the **Emission** tab.
2. Mark the data and copy it via *Ctrl+C*. Alternatively, right-click on the marked area to open the shortcut menu and select **Copy**.
3. Change to the **Dye Editor** dialog.
4. On the **Emission** tab, click in the first row of the **Wavelength (nm) | Spectrum value** table an press *Ctrl+V*. Alternatively, right-click in the first row to open the shortcut menu and select **Paste**.
   → The emission data is inserted in the table and the emission graph is displayed next to the table.
5. Save the dye via **File** | **Save...**.

You have saved a new dye in your custom database.

You can use the new dye in the **Smart Setup** or in the **Channels** tool.

6.7.2.3 Copy Data from a Preset Dye

If the data of a preset dye, e.g. the emission data of the DAPI dye resembles your custom dye, you can copy the data from the preset dye into a new custom dye.

NOTICE

Do not edit preset dyes

Editing preset dyes leads to irreversible data loss.
Note that you should therefore only copy the data set of a preset dye.

Prerequisite
- ✓ You have created a new dye in the **Dye Editor** dialog.

1. Open a second **Dye Editor** dialog via **Tools** | **Dye Editor...**.

2. Confirm the occurring dialog with **Yes**.
   Perform the following steps to open the **ZEN Dye Database** in the second **Dye Editor** dialog:
3. In the **File** menu, click on the **Open folder...** entry.
4. In the Windows dialog, open the **DyeDataBase** with all preset dyes by following the file path:
   C:/Program Files/Carl Zeiss/ZEN/ZEN 2 (blue edition)/ZEN/Resources/DyeDataBase
   → All preset dyes are displayed in the second **Dye Editor** dialog.
5. Use the **Search** input field, to quickly find a preset dye with similar values, e.g. DAPI.
6. Select the desired dye.
7. Click on the **Copy the current dataset as XML** button.

   Alternatively, click **Dataset** | **Copy**.
   → The emission data is copied to the clipboard.
8. Change to the first **Dye Editor** dialog, to edit your custom dye.
9. In the **Dataset** menu select **Paste Part** | **Spectra** | **Emission** | **Data**.
   → The emission data from the preset dye is copied into your custom dye.
10. Edit the custom dye emission data, until it matches your requirements.
    → The emission graph is displayed next to the table.
11. Save the dye via **File** | **Save...**.

You have saved a new dye in your custom database.

You can use the new dye in the **Smart Setup** or in the **Channels** tool.

Info

You can paste other properties of preset dyes just as the emission data, e.g. **All Spectra** data or **Emitter Properties**.

6.7.3 Adding a New Dye to the Data Set

Prerequisite

✓ You have successfully created a first custom dye and a custom dye database, see *Creating a Custom Dye [▸ 57]*.

✓ You have opened the **Dye Editor** dialog.

1. In the **Dataset** menu select **New...**.
   → The **Create new dataset** dialog opens.
2. Enter an ID for your new dye, e.g. DyeC2 and confirm with **OK**.
   → The entry **Unnamed other dye** appears in the **Exeml Collection** list.
3. Add a name for the new dye by clicking on the **Plus** icon in the **Names** section.

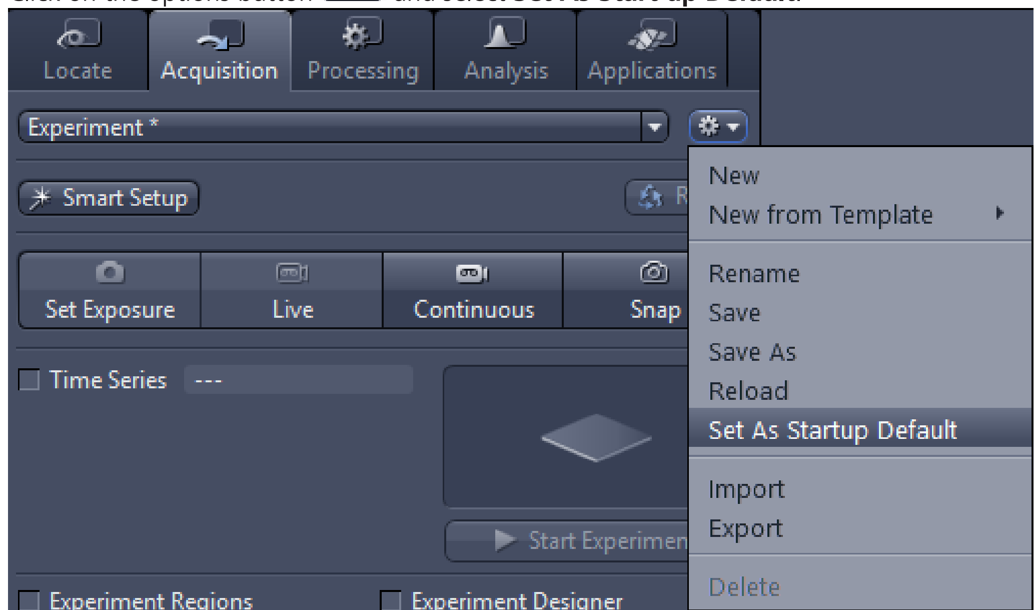
4. Enter a name in the **Name** input field.
5. Edit the properties of the new dye, see
   Type in Data Manually [▸ 58] or
   Copy Data from an Other Source [▸ 58] or
   Copy Data from a Preset Dye [▸ 58].

6.8 Setting an experiment as startup default

Any experiment can be set as a startup default indicated by a symbol.

If you want to set an experiment as startup default, the following steps are necessary.

1. In the menu bar click on **Tools > Options...**.
2. Click on **Startup/Shutdown**.
3. In the **Experiment** section, select **Load Default Experiment** from the drop down list.
4. Click on **OK**.
   → The **Options** dialog closes.
5. Configure your experiment for startup default or select an existing experiment.
6. Click on the options button and select **Set As Start up Default**.



The actice experiment is set as startup default now and marked with the symbol.

This experiment is loaded when starting the ZEN software.

6.9 Creating default experiments as templates automatically

It is possible to generate an experiment template. This cannot be modified but used as a starting point for your acquisition.

If you want to generate an experiment template, the following steps are necessary.

1. Create an experiment.
2. In the menu bar, click on **Help > About ZEN...**
   → The **About ZEN** dialog appears.
3. Click on **Show ZEN Information**.
   → The **Application Information** dialog appears.
4. Open the **Folders** tab, scroll to the **Experiment Templates** entry, and double click on it.
   → The folder **Experiment Setups** will open.
5. Export the experiment generated before into the **Carl Zeiss\ZEN\Templates\Experiment Setups** folder.
6. Create a descriptive name for the experiment template.

You have successfully created an experiment template.

It is possible to generate and save multiple templates.

To apply an experiment template, see chapter *Applying an experiment template [▸ 61]*.

6.10 Applying an experiment template

If you want to apply an experiment template as a starting point for your acquisition, the following steps are necessary.

Prerequisite ✓ The experiment template to be used has been created before, see chapter *Creating default experiments as templates automatically [▸ 60]*.

1. On the acquisition tab, click on the options button and select **New from Template**.
   → The available experiment templates will be shown on the right side.
2. Click on the name of the appropriate template.

The selected experiment template will be loaded as starting point for the acquisition.

6.11 Adding Annotations to Images or Movies

You can add annotations to visualize a specific region or to add metadata or specific measurement information to images and movies.

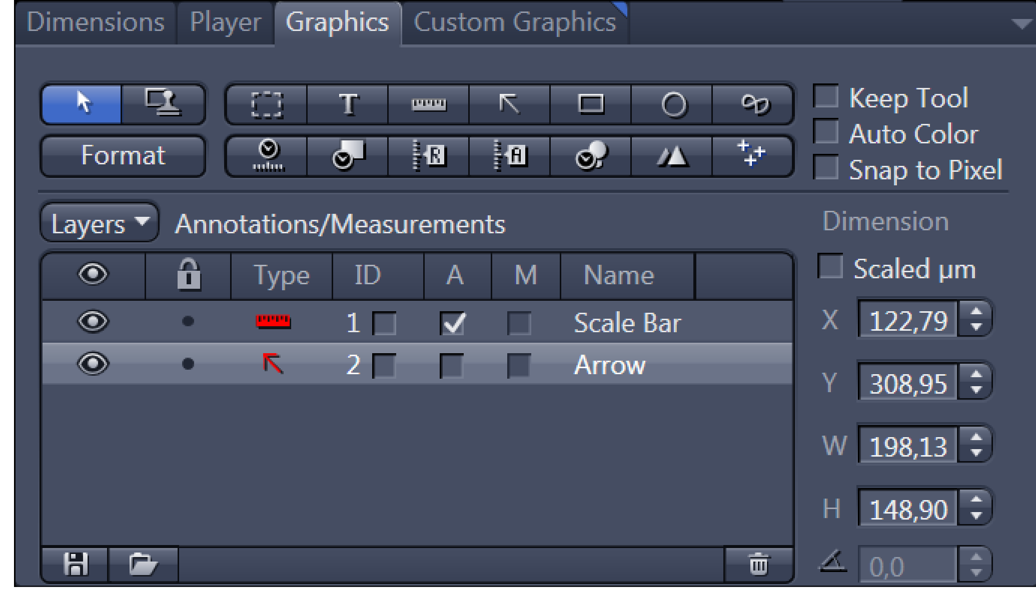
You can use the annotations within several workflows and functionalities:

- For your own information, you can display metadata to images and movies.
- When working with mean ROI, you use the annotation functions to draw regions into the image.
- When analysing images manually, you can record your measurement results in the image.
- When exporting images or movies, you can add annotations that are visible in the export format of your images and movies.

You can use the annotation functions on the **Graphics** tab as well as in the **Graphics** menu. For information on the **Graphics** menu, see *Graphics Menu [▸ 620]*.

Prerequisite ✓ You have acquired or loaded an image or a movie.

1. In the **Center Screen Area** select the **Graphics** tab.



2. Click on one of the icons to add an annotation or a measurement.

Remove Annotations

1. Select the annotation in the **Image** view or in the **Selection** table, and press the *Del* key. Alternatively, right-click the annotation and select **Delete**.

Additionally, the annotations can be edited, rearranged, and formatted. For more information, see *Editing Annotations in Images or Movies [▸ 62]*.

See also

- Graphics Tab [▸ 894]
- Custom Graphics Tab [▸ 901]

6.12 Editing Annotations in Images or Movies

You can edit, rearrange and format annotations in an image or movie.

Edit Annotations

You can change the annotations type or the unit of the time or of the measurement.

1. To edit text in a textbox, double-click the textbox and change the text.
2. In the image, select the annotation and right-click to open the context menu. Select **Format Graphical Elements**.
   → The **Format graphical elements** dialog opens.
3. Edit the annotation and click **Close** to save.

Rearrange Annotations

1. Select the annotation with the left mouse button and drag and drop the annotation to another position.

Format Annotations

1. In the image, select the annotation and right-click to open the context menu. Select **Format Graphical Elements**.
   → The **Format graphical elements** dialog opens.
2. Format the annotation and click **Close** to save.

See also

- Format Graphical Elements Dialog [▸ 898]
- Adding Annotations to Images or Movies [▸ 61]

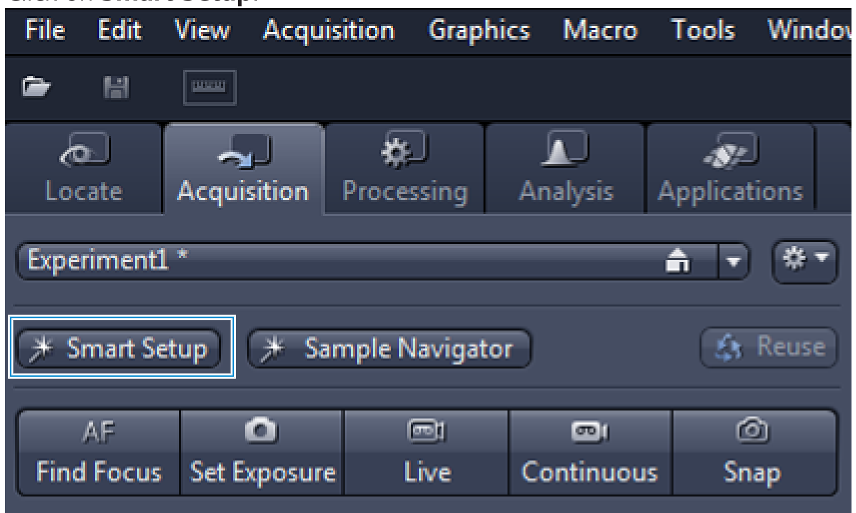
7 Confocal Image Acquisition (LSM)

7.1 Acquiring Confocal Images

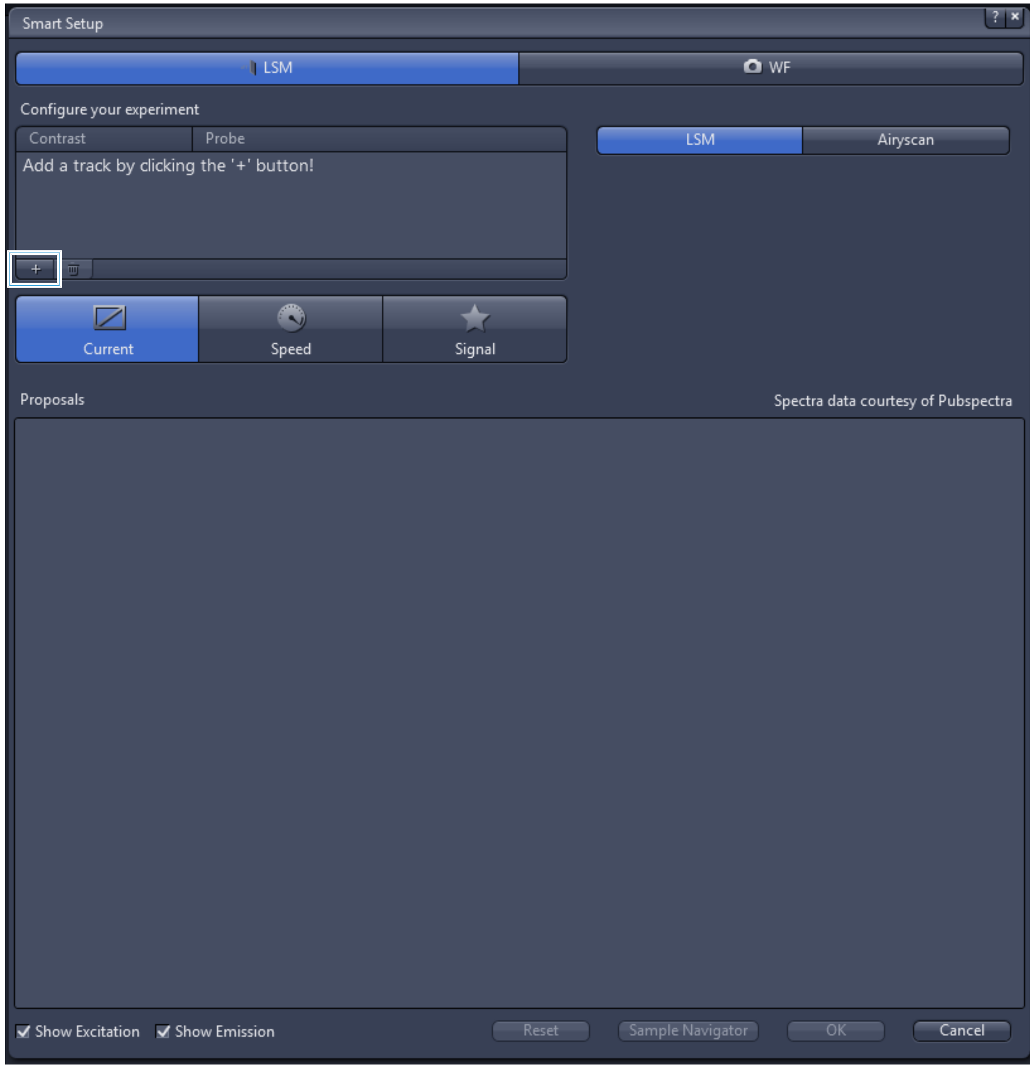
To acquire confocal images you first have to setup the acquisition parameters and configure your experiment. Therefore we recommend to use **Smart Setup** as this will automatically give you certain suggestions for the experiment configuration, e.g. Airyscan acquisition or camera based acquisition. In the following guide you will learn how to use Smart Setup and acquire a first confocal image. Because there`s a huge variety of samples (and suitable experiment configurations) this guide shows the necessary basics only.

Prerequisite ✓ You are on the **Acquisition** tab.

1. Click on **Smart Setup**.

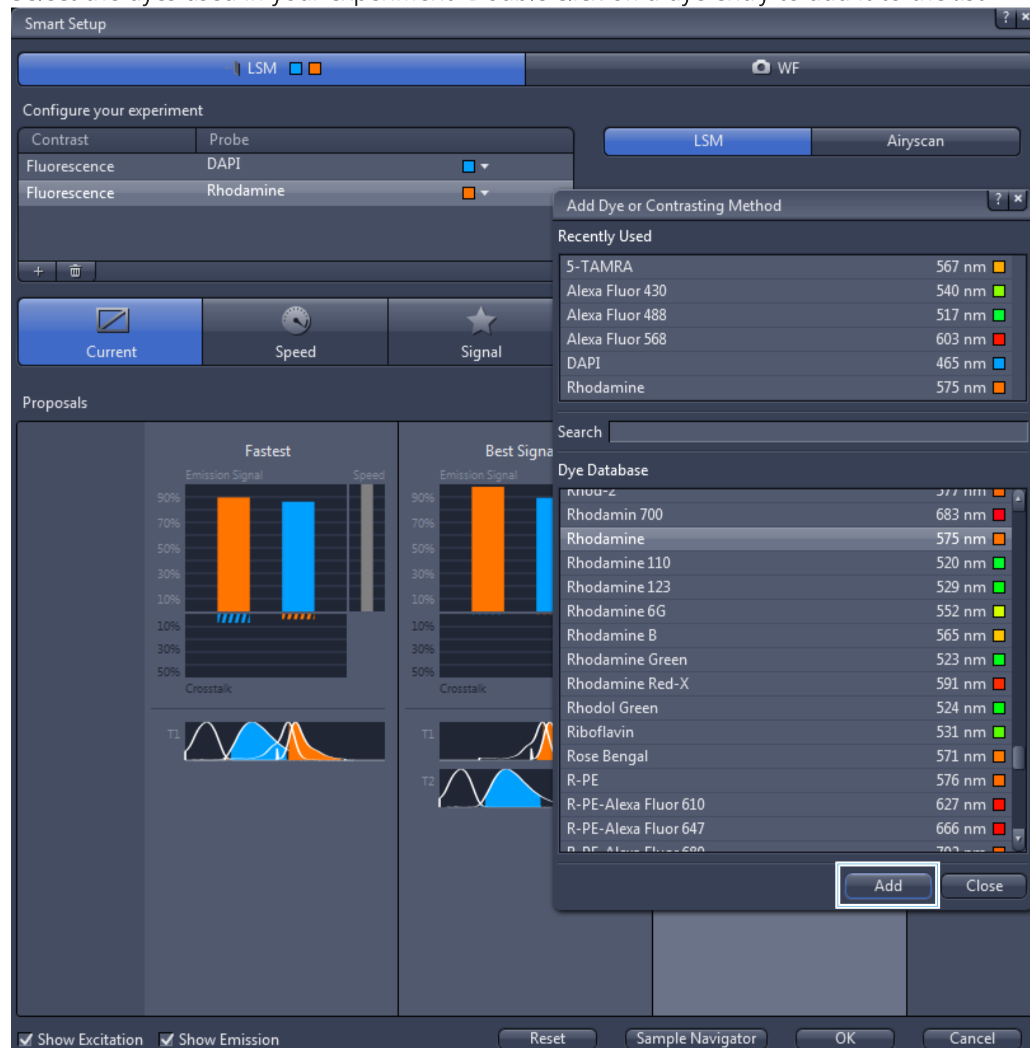


2. Click on + **Add**.

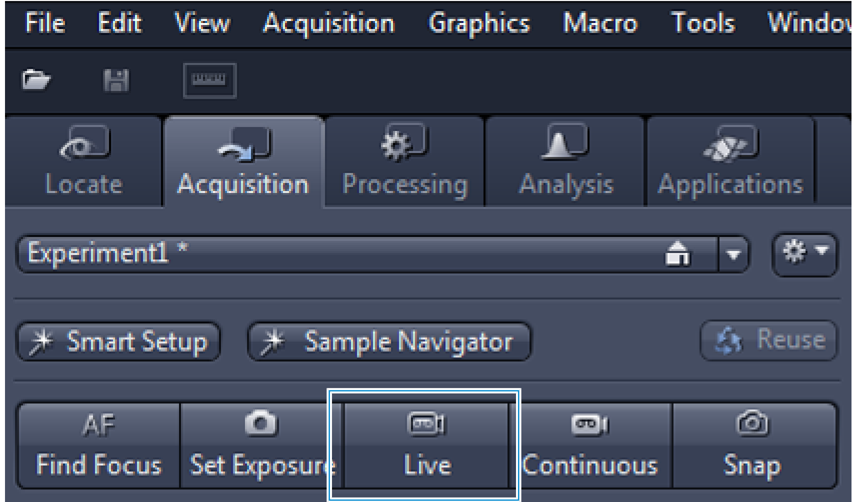


→ The **Add Dye** dialog opens.

3. Select the dyes used in your experiment. Double click on a dye entry to add it to the list.



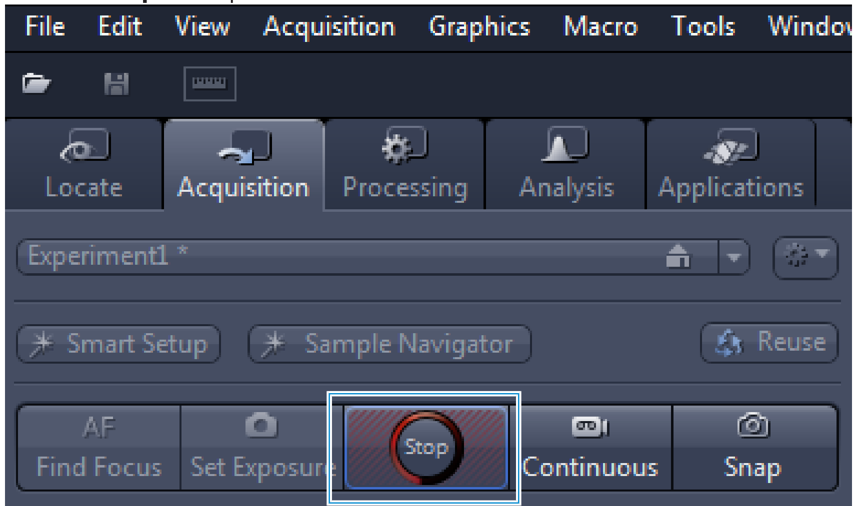
4. Click on **Ok** to close Smart Setup.
5. Make sure that the cover glass of you sample points towards the objective. Use the appropriate immersion medium for the objective.
6. Click on **Live** to start a live scan.



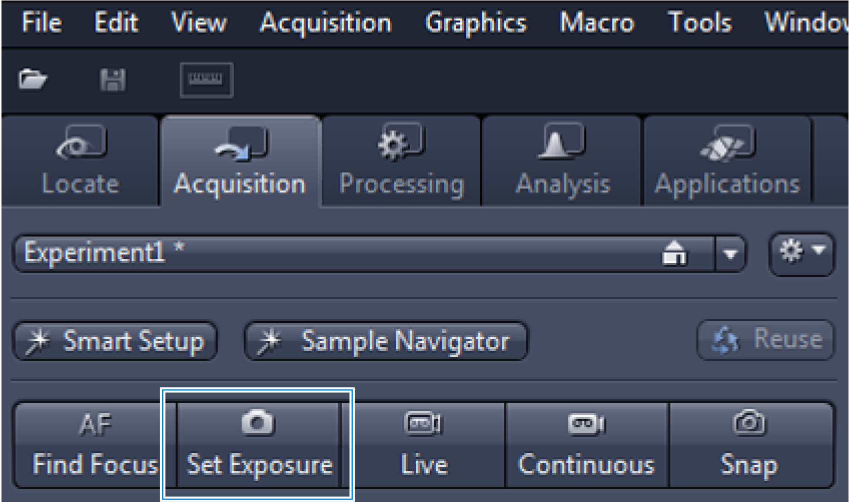
→ You will see the image of your sample in the center screen area.

7. Search the desired sample area and focus with the joystick. Alternatively use the mouse wheel while pressing the *Ctrl* key.

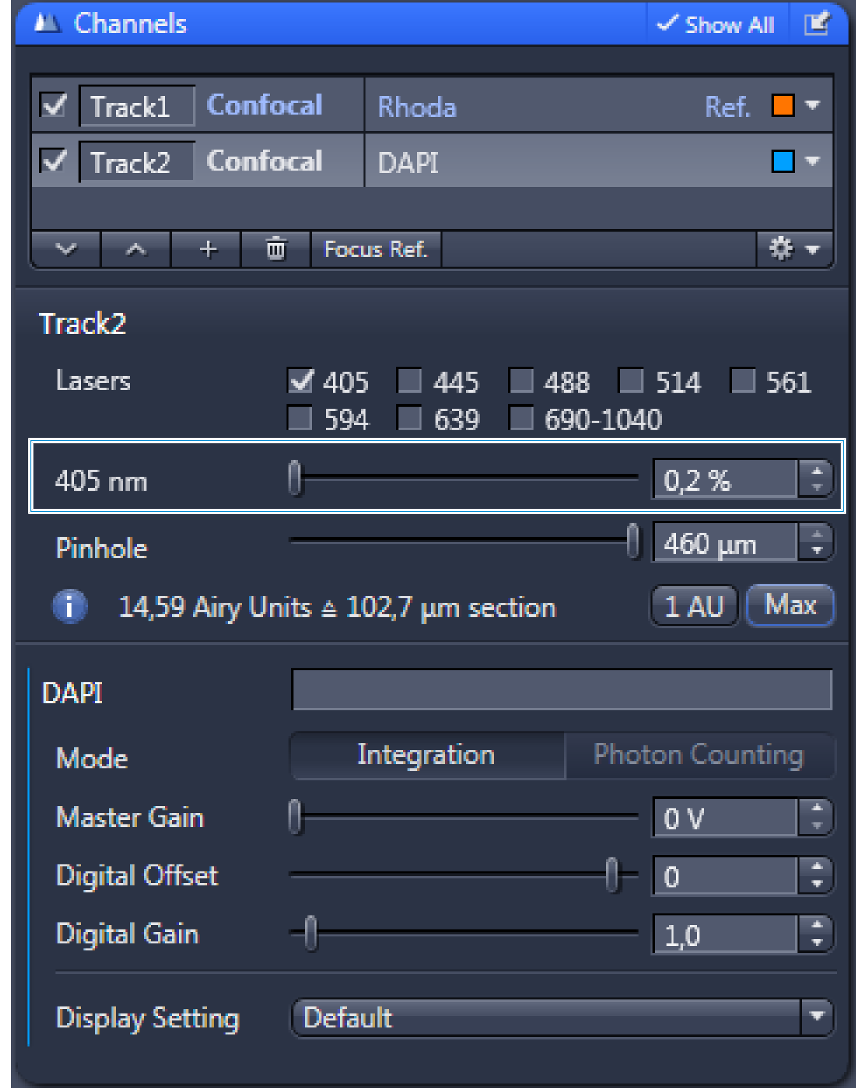
8. Click on **Stop** to stop the live scan.



9. Click on **Set Exposure** to automatically adopt the sensitivity of the detector to the sample brightness.

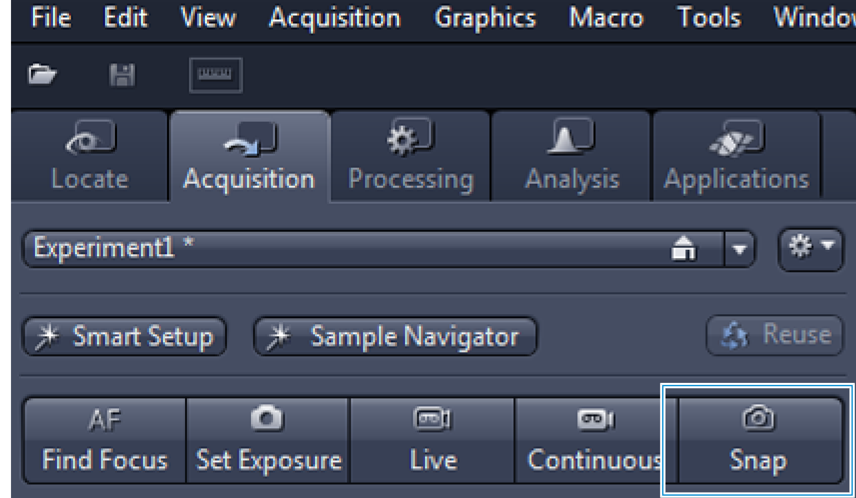


10. **Optional:** If you work with weakly stained samples you can try to increase the laser power under **Channels** | **Lasers**. Then repeat the last step by clicking on **Set Exposure**.



→ The sample is adjusted correctly.

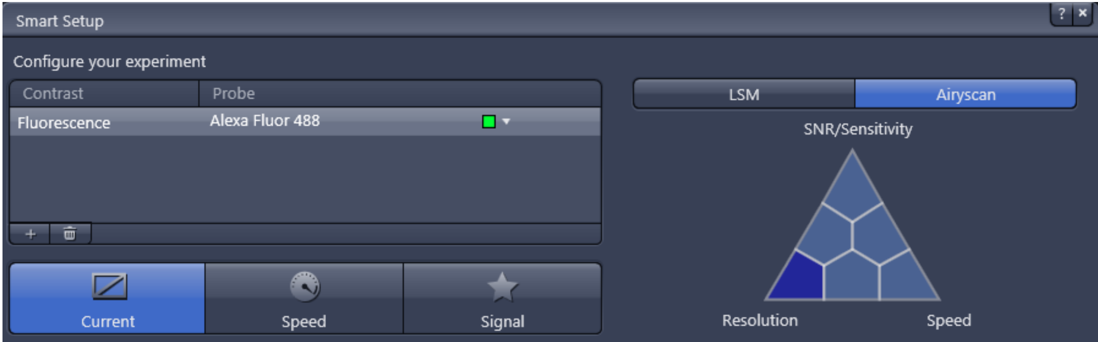
11. Click on **Snap** to acquire a single confocal image.



You have successfully acquired a confocal image. Save the image in the **Images and Documents** tool or under **File** | **Save**.

7.2 Using Smart Setup with Airyscan LSM 980 and LSM 900

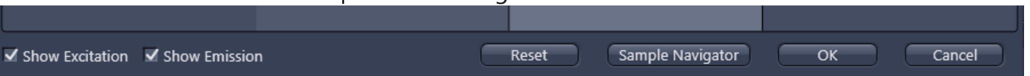
After selecting samples and overall preference for speed (single track, more crosstalk) or signal (multi track, less crosstalk), you can choose LSM confocal or Airyscan 2 detection modes in **Smart Setup**.



LSM confocal acquisition

Prerequisite ✓ **Smart Setup** is open.

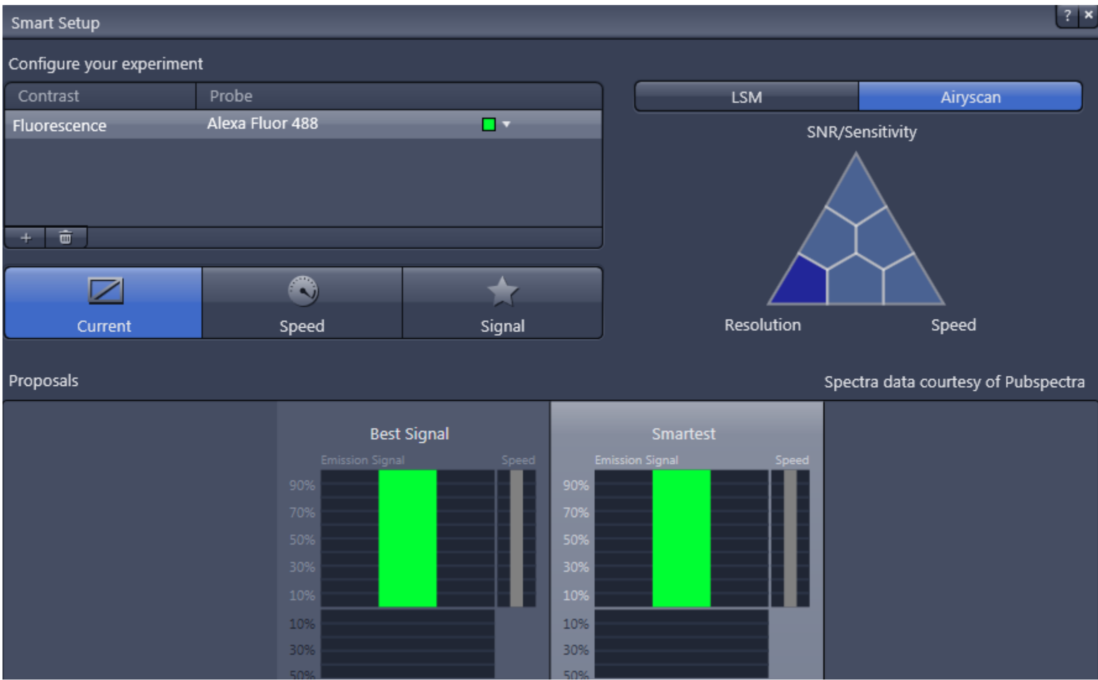
1. Select **LSM** at the detection selection on the right.
2. Click on **OK** to activate the acquisition settings.



Airyscan 2 detection mode

Prerequisite ✓ **Smart Setup** is open.

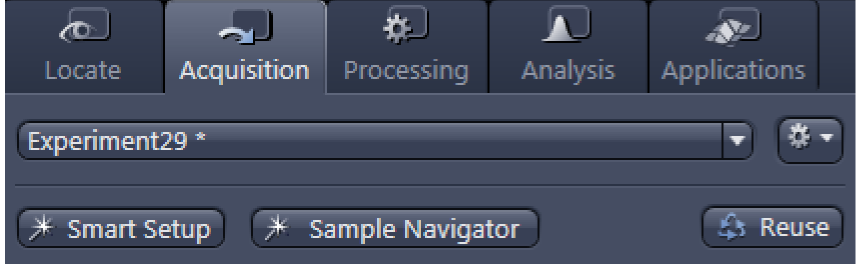
1. Select **Airyscan** at the detection selection on the right.
2. Click into the triangle depending on your preference for resolution, speed and SNR/sensitivity.
3. Click in one of the areas in the lower left corner to activate available modes with super resolution image quality.
   Depending on how close the selected area is to **Speed**, the amount of parallelization in pixel acquisition will increase.
4. Click in one of the areas on the line at the right between **SNR/Sensitivity** and **Speed** to activate modes with at least confocal image quality. Depending on how close the selected area is to **Speed**, the amount of parallelization in pixel acquisition will increase.



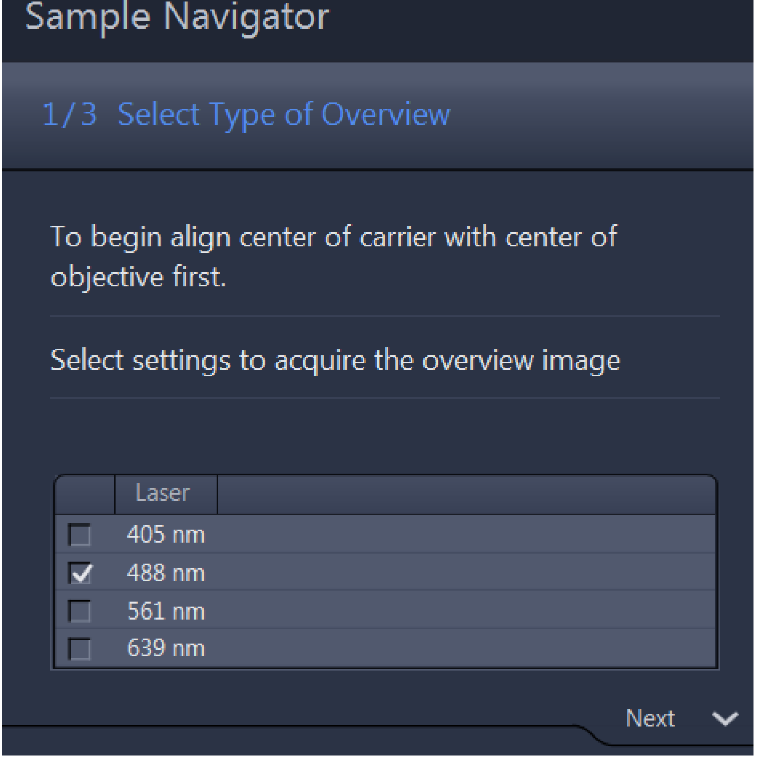
7.3 Using the Sample Navigator with LSM 980 and LSM 900

The **Sample Navigator** wizard is a tool to find the focus plane and quickly acquire an overview scan of your sample. You can also simplify the search for a region of interest for the actual imaging experiment. Together with **Smart Setup**, it allows to set all basic settings for a new sample without extensive search of the sample by the eyepieces.

1. In the **Acquisition** tab, click on the **Sample Navigator** button.

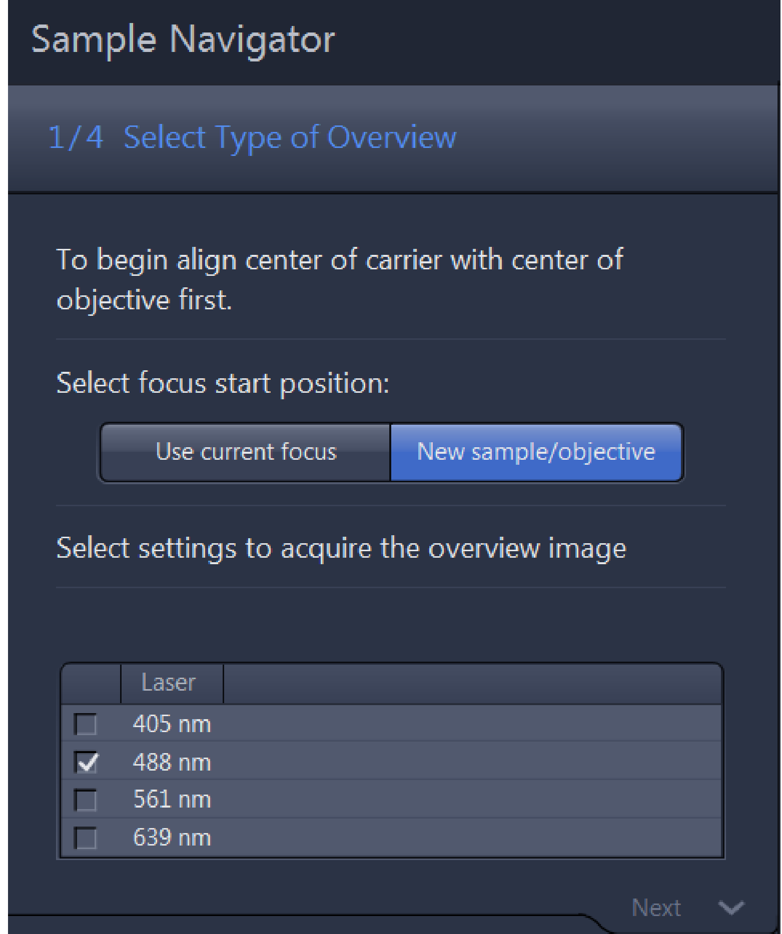


→ A wizard shows up which guides you through the required steps. On the left you find general settings, in the middle temporary information and steps to do.



2. Put a sample on the stage and center the sample with some structure over the center of the objective.
   The preferred objective to use is a 2.5x overview objective. Alternatively, 5x and 10x objectives can be used if a **Axiocam** is available on your system. If no Axiocam is present, the LSM T-PMT will be used for detection.

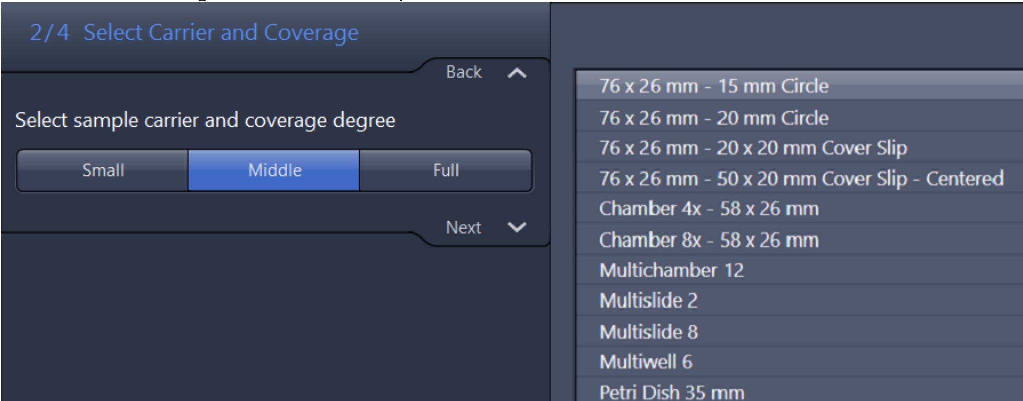
3. Select a checkbox with the wavelength that matches the label of your sample.



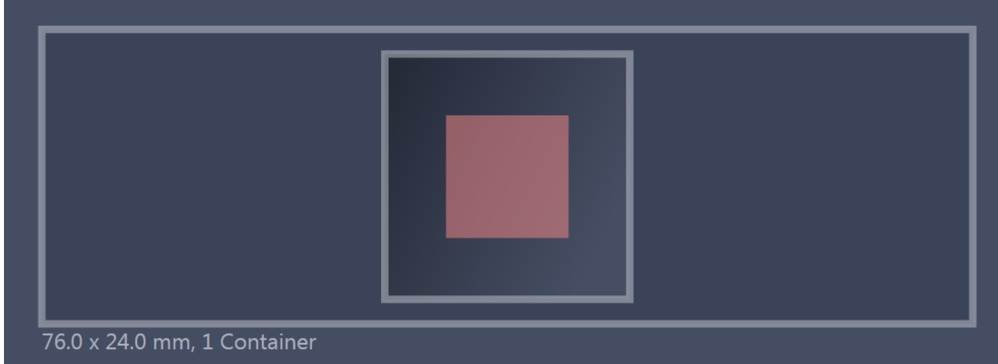
→ You are asked whether this sample was already focused, or if a new sample was put on the microscope. With a new sample, you are asked to focus the objective a bit closer than the putative focus plane. This is because the autofocus will move away from the sample when searching for the focus plane.

4. Click on **Objective is now close to the sample** to confirm.

5. Click on **Next** at the bottom of the wizard.
   → This guides you to Step 2, which selects the sample carrier
6. Select a matching sample carrier type from the selection list in the middle.
   The coverage degree can be selected with the three buttons **Small**, **Middle** and **Full**. **Full** means full coverage of the Cover slip area.



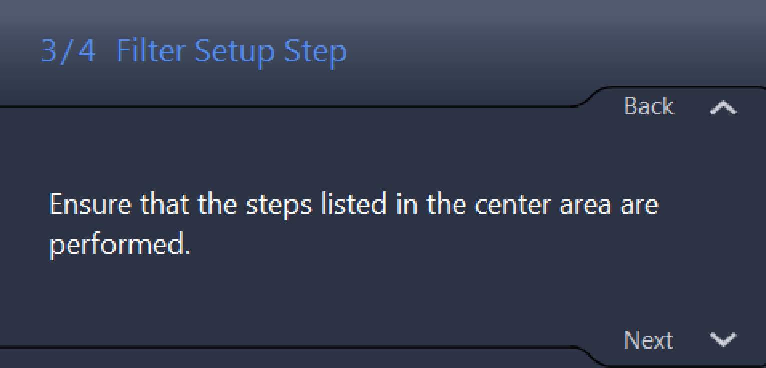
   → The selected coverage is displayed by a red area on the sample carrier drawing.



7. Click on **Next** at the bottom of the wizard .

   → This guides you to Step 3, which selects the Kondensor Filter in case the T-PMT was selected as detector.

8. Since **Sample Navigator** uses transmitted fluorescence with the T-PMT, a laser blocking filter needs to be inserted. This filter is located in one of the DIC positions of the condenser, and labelled T-FL. Also, correct Köhler adjustment is needed. Setups with **Sample Navigator** have a line marking at the condenser carrier, which helps to find the right position.

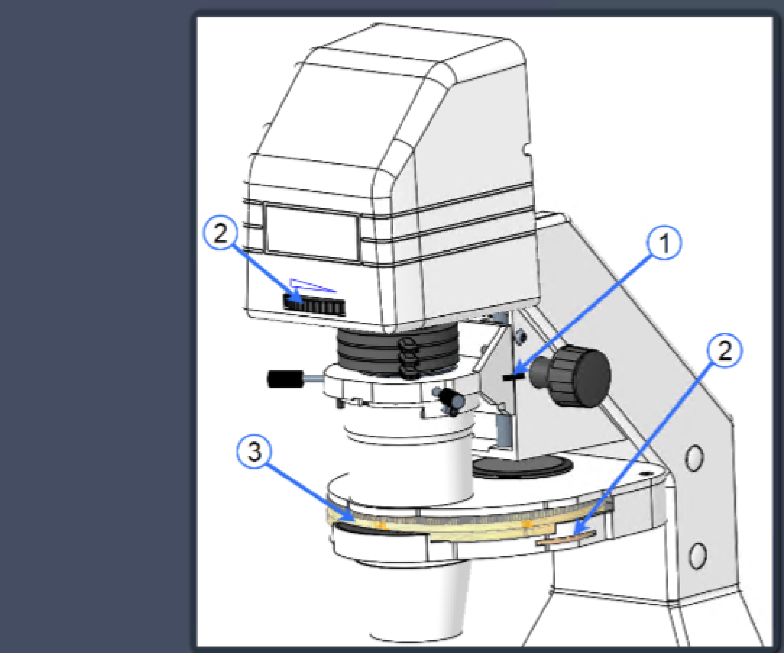


1. Align the lines on the right side of the condenser carrier.

2. Open apertures.

3. Move condenser position to T-FL.

4. Use the black cloth to protect the sample space from room light.



9. Click on **Next** at the bottom of the wizard .

→ This guides you to Step 4, which is the autofocus step.

10. Click on **Focus Detection** to start the autofocus, and observe the image in the middle.

Sample Navigator

4/4 Focus Step

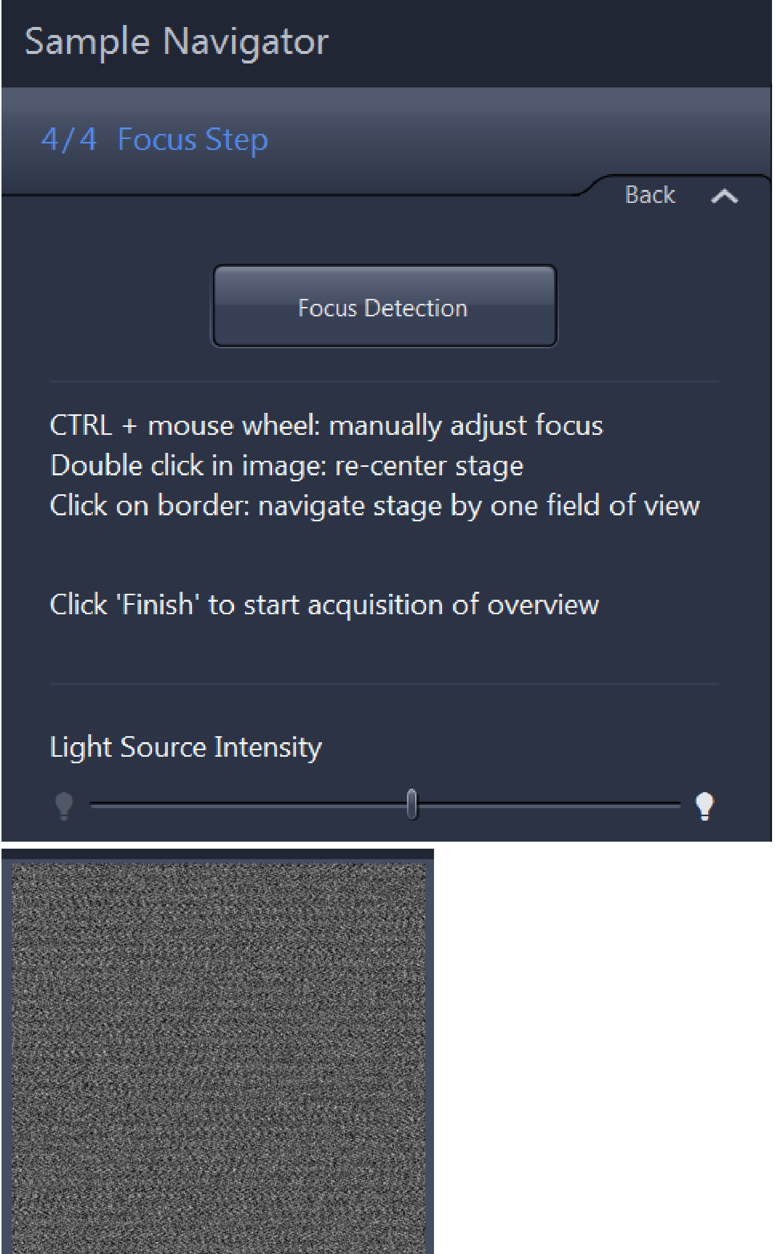
Back

Focus Detection

Click 'Focus Detection' to start autofocus.

Focus step

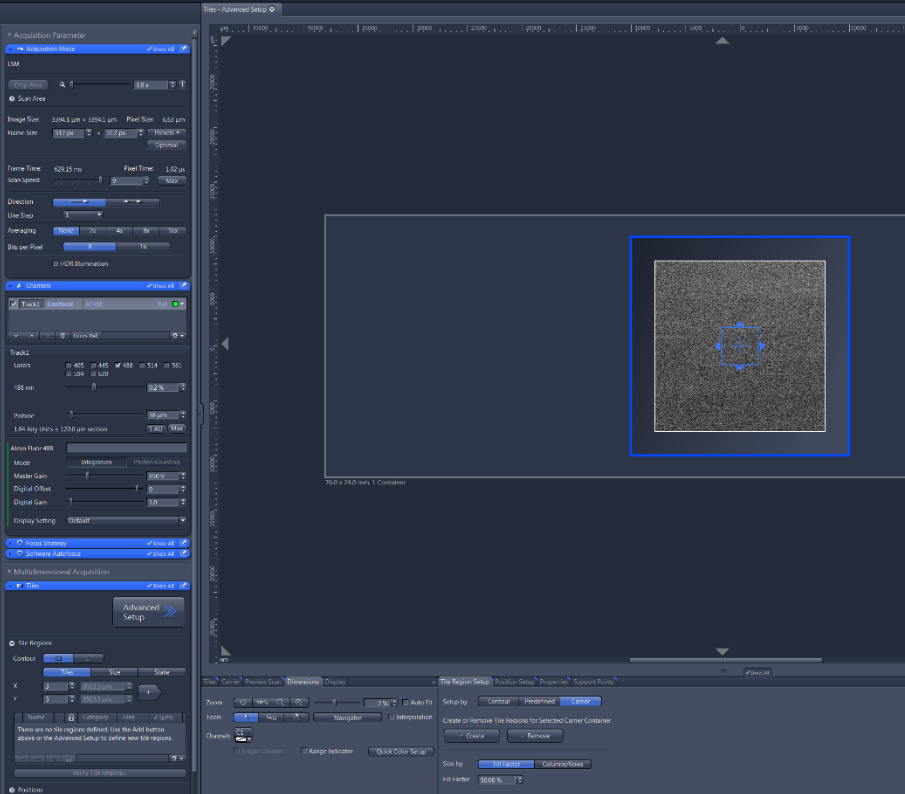
11. If the image is over- or underexposed, the light intensity needs to be adjusted with the slider **Light Source Intensity**.



12. In case the autofocus result is not suffient, correct it by manually focussing the sample with the focus drives or *CTRL*+mouse wheel.
13. Click on **Finish** to start the automatic overview tile scan.

The wizard is closed and a new image container opens.

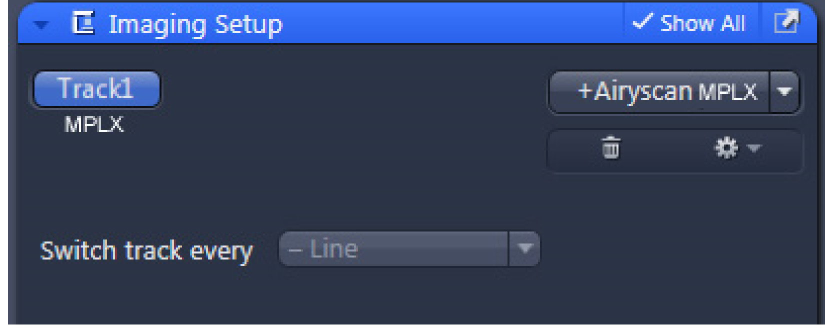
The overview image can be used to navigate to ROIs, by a double klick on an interesting structure. This will re-center the stage. Proceed with normal image acquisition by setting up acquisition parameters for a higher magnification objective.



7.4 Acquiring LSM 900 images with Airyscan 2 multiplex modes

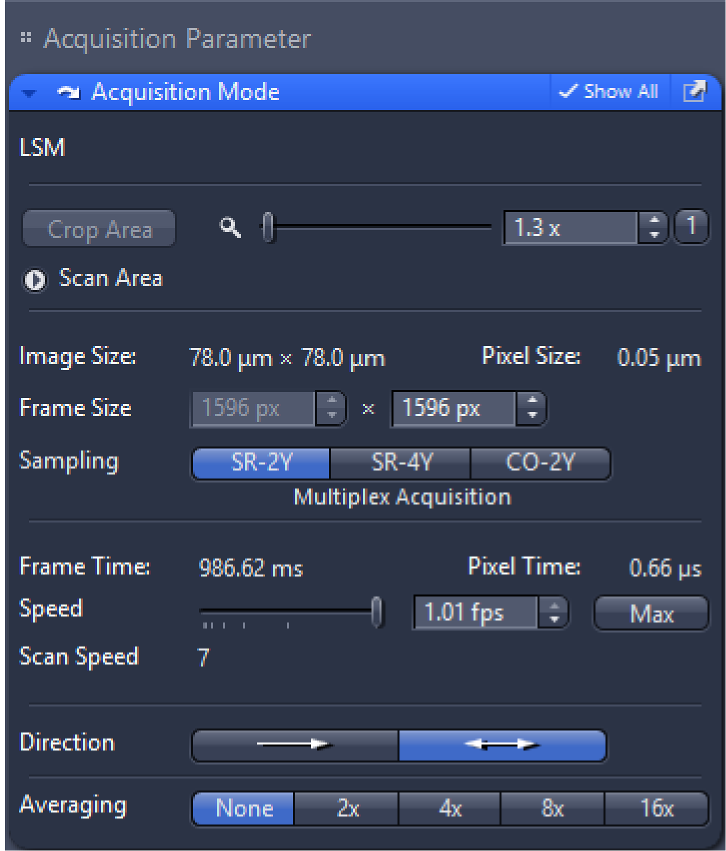
Multiplex modes allow a faster image acquisition with Airyscan 2 detectors. The acquisition is parallelized in the Y-direction, which allows to process full SR or confocal resolution though not every line in Y was scanned during acquisition. The Airyscan 2 detector has nonetheless acquired data for all lines in the image.

1. On the **Acquisition** tab, in **Imaging Setup** select **Airyscan MPLX** in the drop down list.

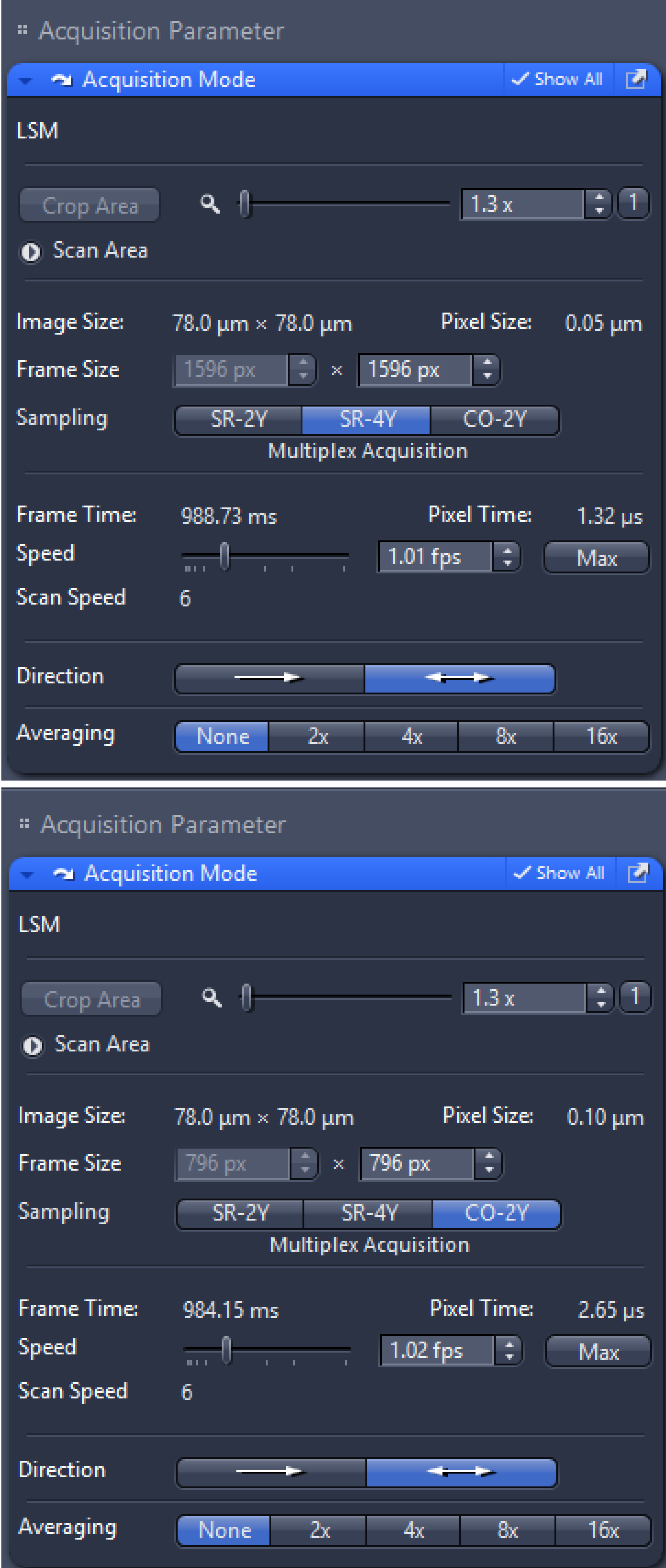


→ The respective Airyscan mode menu will be displayed in the **Acquisition Mode** tool.

2. Click on **SR-2Y** to activate a parallelization of 2 lines in the Y-direction. Alternatively, if no super resolution is required, click on **CO-2Y**.



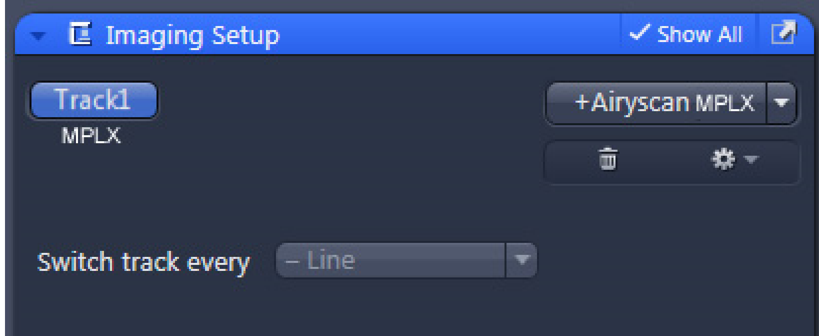
3. If 2x parallelization is not fast enough, click on **SR-4Y** to activate a parallelization of 4 lines in the Y-direction. This mode always delivers super resolution results. For confocal resolution, click on **CO-2Y**.



7.5 Acquiring LSM 980 images with Airyscan 2 multiplex modes

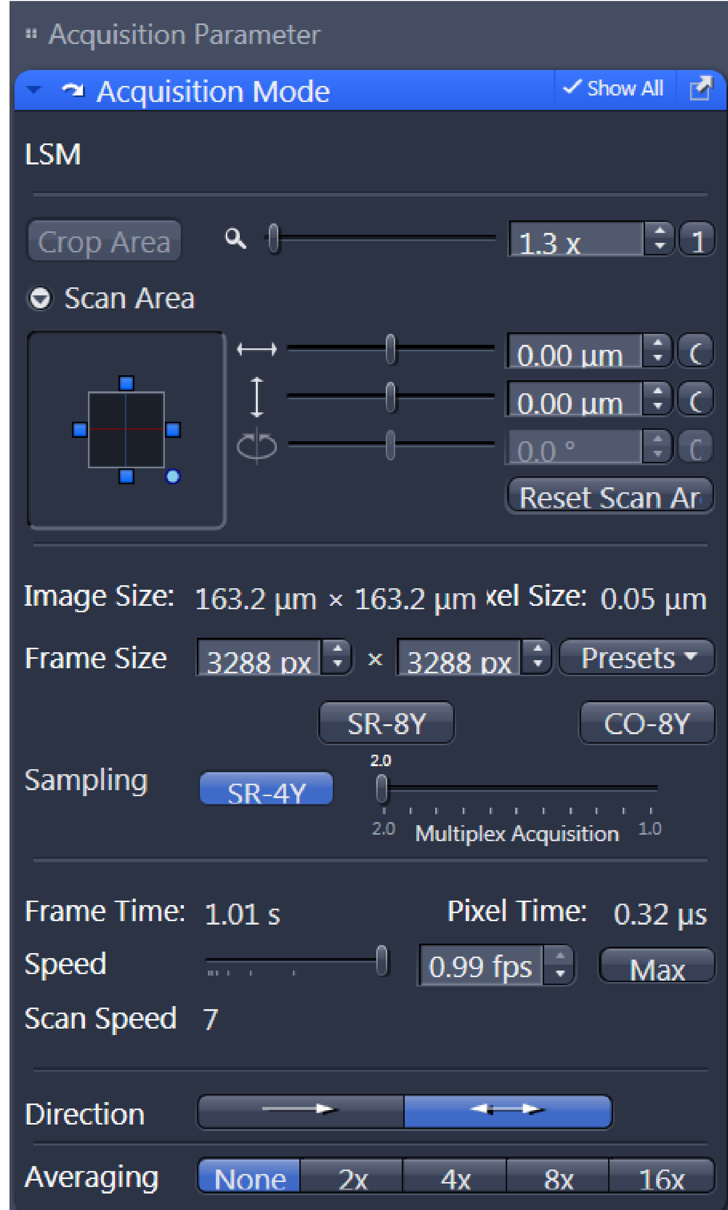
Multiplex modes allow a faster image acquisition with Airyscan 2 detectors. The acquisition is parallelized in the Y-direction, which allows to process full SR or confocal resolution though not every line in Y was scanned during acquisition. The Airyscan 2 detector has nonetheless acquired data for all lines in the image.

1. On the **Acquisition** tab, in **Imaging Setup** select **Airyscan MPLX** in the drop down list.

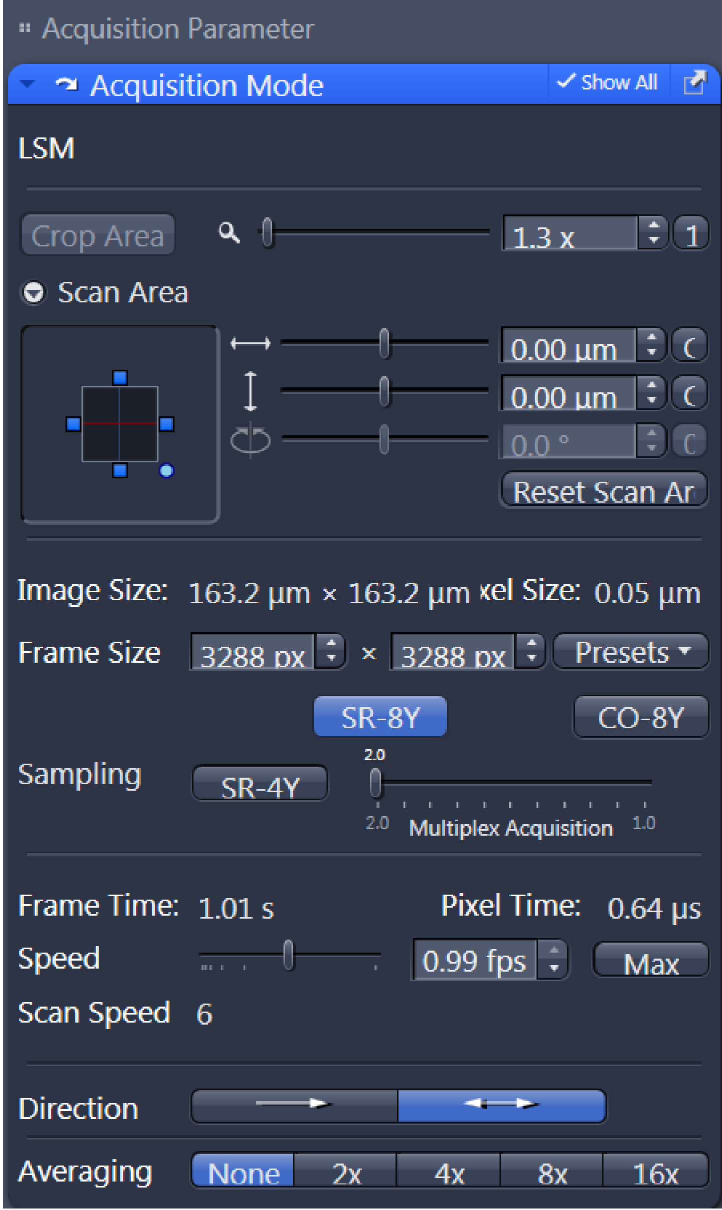


→ The respective Airyscan mode menu will be displayed in the **Acquisition Mode** tool.

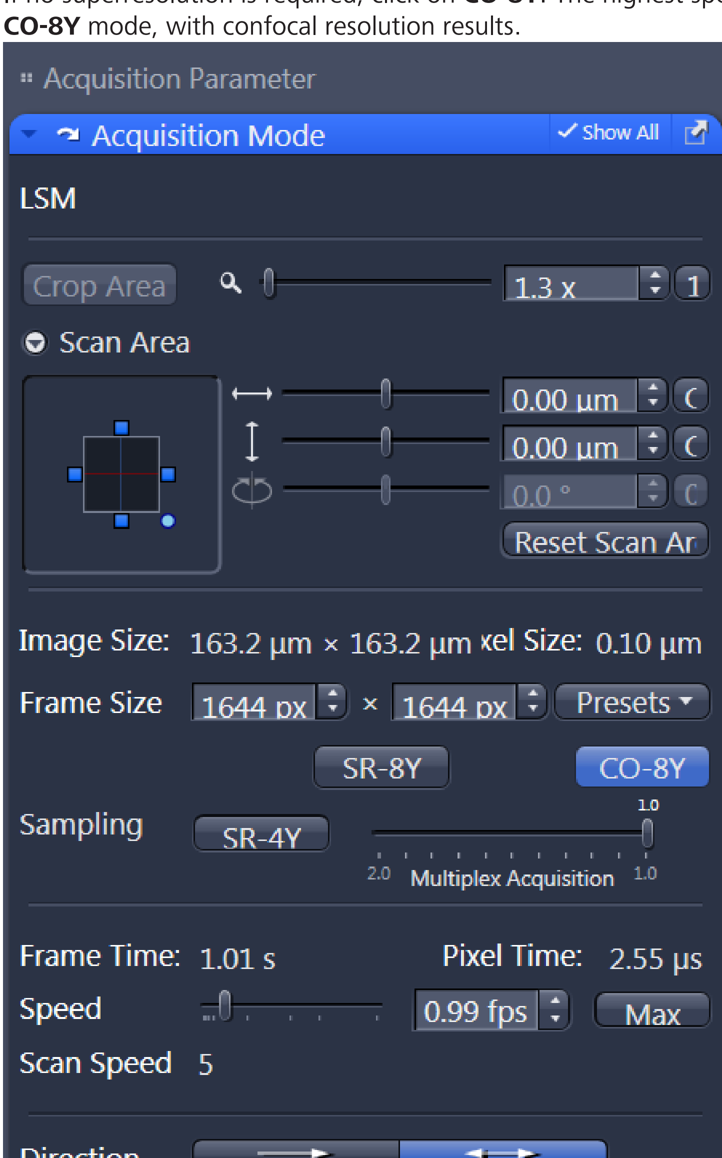
2. Click on **SR-4Y** to activate a parallelization of 4 lines in the Y-direction.



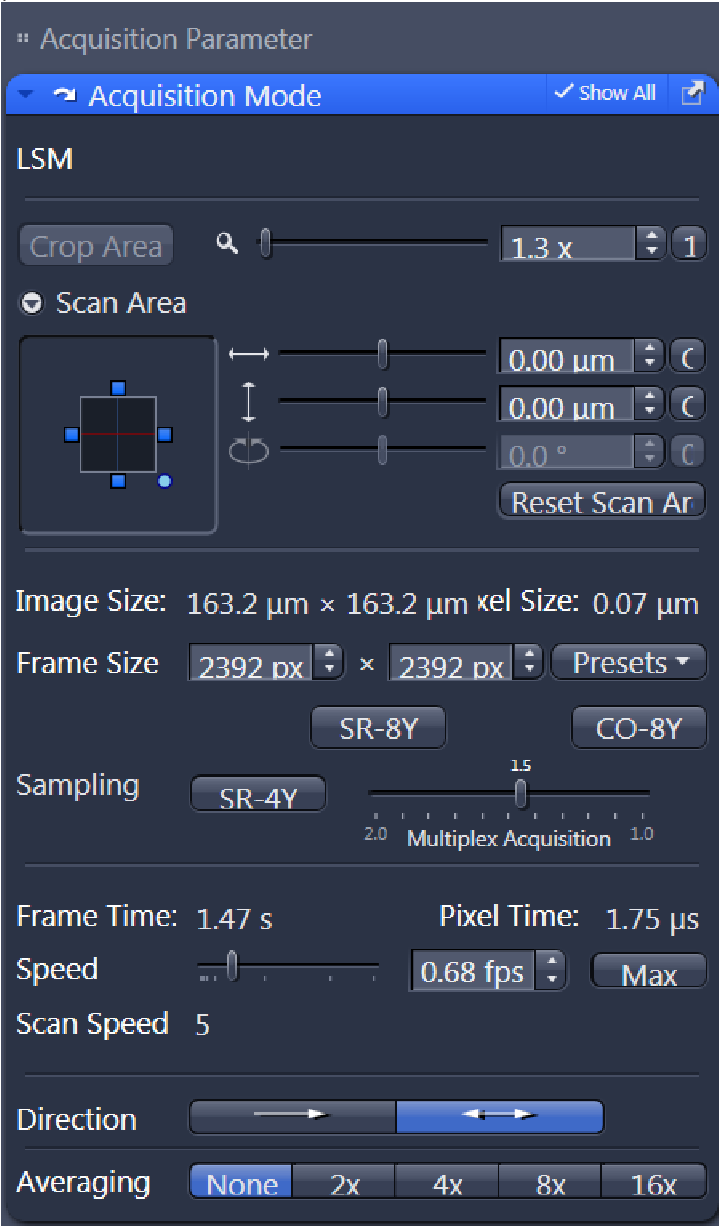
3. If 4x parallelization is not fast enough, click on **SR-8Y** to activate a parallelization of 8 lines in the Y-direction. This mode always delivers super resolution results.



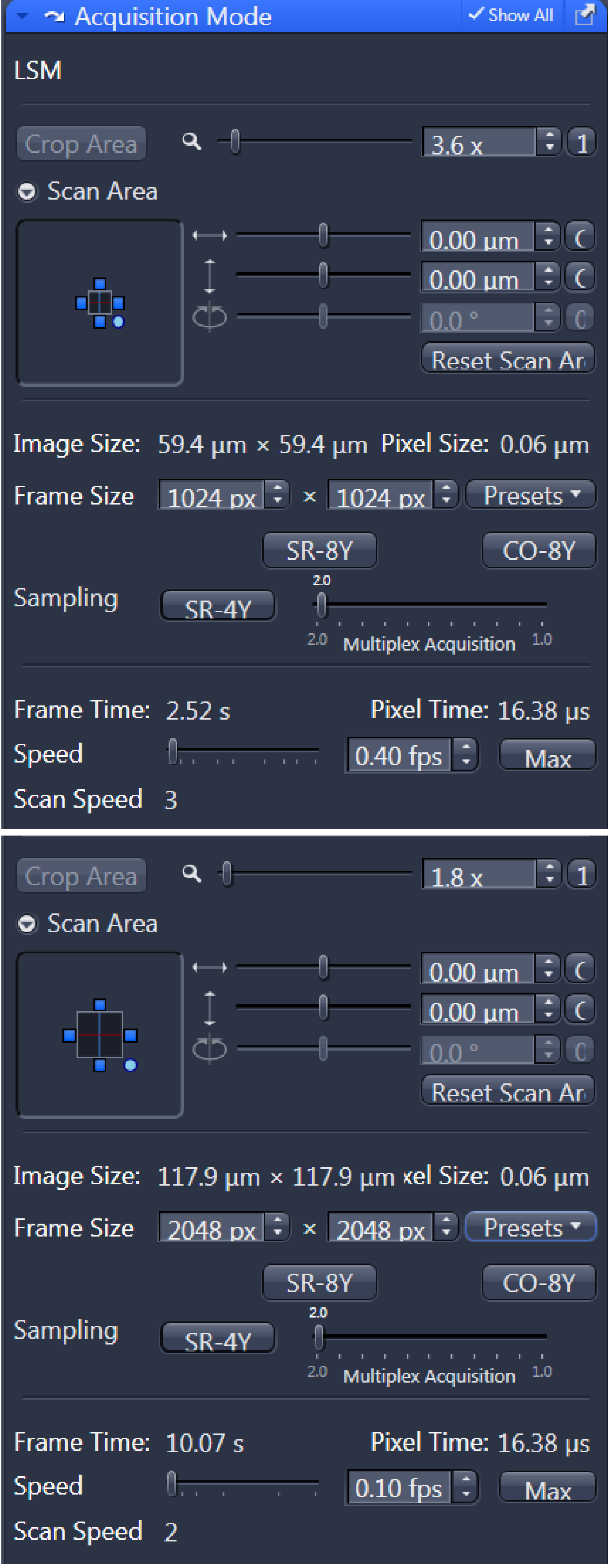
4. If no superresolution is required, click on **CO-8Y**. The highest speed can be selected in **CO-8Y** mode, with confocal resolution results.



5. The slider between these buttons allows to select any sampling between these sampling points.



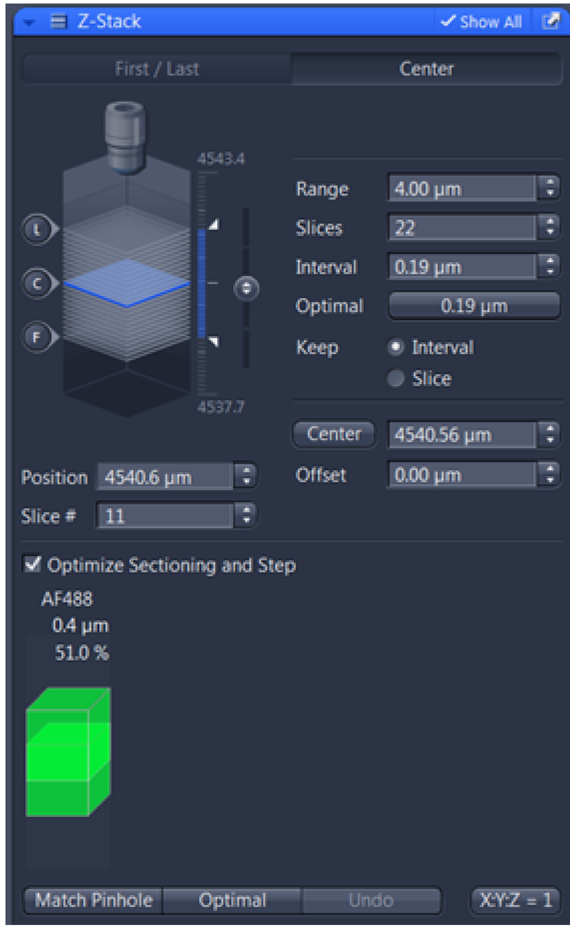
6. It is also possible to select a desired image format, e.g. 1024x1024 pixels. The slider will then display the sampling factor deriving from the selected zoom or crop area.



Additional information for using Z-stacks:

The Z stepping is not automatically adjusted to the sampling mode, but is set to secure maximum resolution. The button **Optimal** and the checkbox **Optimize Sectioning and Step** in the **Z-Stack** menu set the acquisition for optimal oversampling in Airyscan SR resolution.

Faster volume speed can be achieved by intentionally selecting a wider Z stepping e.g. for **CO-8Y** mode. The recommendation for a fast **CO-8Y** stack would be 1.5x – 2x the calculated optimal interval. Deselect the check box **Optimize Sectioning and Step** to choose your interval manually.



8 Working with Focus Strategies

8.1 Introduction

If you want to work with focus strategies you have to use the **Focus Strategy** tool. There you can select the suitable strategy and adjust the corresponding settings, e.g. defining Z-positions manually or automatically and update these during the experiment. Note that the availability of certain focus strategies depends on your system and available components (e.g. Definite Focus.2).

General Preparations

Prerequisite

- ✓ To use focus strategies, you will need a motorized focus drive/Z-drive.
- ✓ You are in the **Left Tool Area** on the **Acquisition** tab.
- ✓ You have *created a new experiment [▸ 44]*, *defined at least one channel [▸ 43]* and adjusted the focus and exposure time.

1. Activate the acquisition dimensions (e. g. **Tiles**, **Time Series**) that you want to use for your experiment.
2. Open the **Focus Strategy** tool.

   → The available focus strategies are displayed in the dropdown list.

   → The number of focus strategies available depends on the activated acquisition dimensions (e.g. tiles, time series), the available hardware devices (e.g. definite focus) present, and software license (e.g. **Software Autofocus** and **Tiles** module allow additional focus strategies).
3. Decide which strategy is best suited to your experiment. In case of a **Tiles** experiment the software will automatically select the most appropriate focus strategy if you have not previous selected one. For a detailed description of all strategies, read the chapter *Focus Strategy Tool [▸ 711]*.

You have successfully completed the general preparations. Now follow one of the following sets of instructions for specific focus strategies.

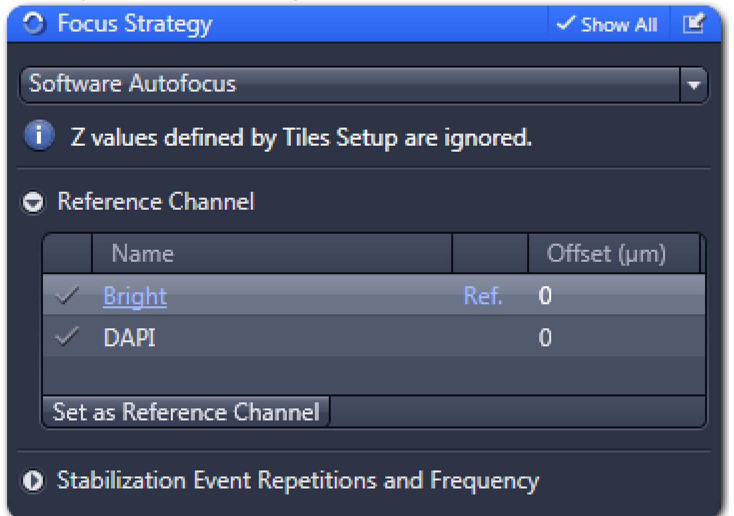
8.2 Using Software Autofocus

Select this focus strategy to automate the focusing of your specimen before and during acquisition with the help of the **Software Autofocus**. This is particularly useful for **Time Series** or **Tiles** experiments.

Prerequisite

- ✓ To use the **Software Autofocus** focus strategy, you will need the module.
  For LSM systems, the **Autofocus** module is part of the system license.
- ✓ You have completed the *general preparations [▸ 86]* for using focus strategies (experiment created, at least one channel defined, acquisition dimensions activated).
- ✓ You are on the **Acquisition** tab in the **Focus Strategy** tool.

1. Select the **Software Autofocus** entry from the dropdown list. Note that the Z values defined by **Tiles Setup** are ignored.



2. In the **Reference Channel** section select the channel that you want to use for the focus action from the list. Expand the section if you don`t see it in full. Note that the reference channel does not necessarily have to be a acquisition channel. Not all acquisition modes can be used as reference. For LSM 980 LSM Lambda tracks and Online Fingerprinting cannot be selected as reference channels. Also, Airyscan SR and MPLX tracks might fail when using such tracks as reference for reflex autofocus function.
3. Click on the **Set as Reference Channel** button.
4. In the **Time Series Loop** and/or **Tiles Loop** sections of the **Focus Strategy** tool you can define when focus actions should be performed during the course of the experiment.
5. Open the **Software Autofocus** tool.
6. Adjust the autofocus settings (e.g. **Quality**, **Sampling**, etc.) to your experiment conditions or use the default settings first.
7. Set up your tile and/or time series experiment.
8. To start the experiment, click on the **Start Experiment** button.

You have successfully used the Software Autofocus to bring images into focus automatically during the experiment.

8.3 Using Definite Focus in Time Series Experiments

Select this focus strategy to use the definite focus device to stabilize the focus in the event of temperature fluctuations during your **Time Series** experiments.

Prerequisite

- ✓ To use the **Definite Focus** focus strategy, you will need the **Definite Focus** hardware device.
- ✓ You have completed the *general preparations [▸ 86]* for using focus strategies.
- ✓ You are on the **Acquisition** tab in the **Focus Strategy** tool.

1. Select **Definite Focus** as focus strategy from the dropdown list. Note that Z values defined by **Tiles Setup** are ignored.
2. In the **Stabilization Event Repetitions and Frequency** section select **Standard** mode. This mode will use our recommend default settings for stabilization. When selecting the **Expert** mode you can adjust all settings according to your needs.
3. Set up a **Time Series** experiment, see *Acquiring Time Series Images [▸ 49]*.
4. Use the **Live** mode to set the focus position using the focus drive.
5. To start the experiment, click on **Start Experiment**.

→ **Definite Focus** is initialized at the start of the experiment at the current focus position. The focus is then stabilized in accordance with your settings during the time series experiment. You will be reminded to set the focus accordingly prior to the experiment starting. You can do this by navigating to a suitable location (position or Tile region) and starting live or continuous. You can then continue with the experiment or cancel it.

You have successfully used the **Definite Focus** to stabilize the focus during a Time Series experiment.

8.4 Using Local or Global Focus Surfaces

For Local or Global Focus Surfaces you need to select the focus strategy **Use Z Values/ Focus Surface defined in Tiles Setup**. This strategy is selected by default, if you have licensed the **Tiles** module. Then you can acquire tiles images along local or global focus surfaces (tile region specific/position specific) and use the focus strategy for optimal image results.

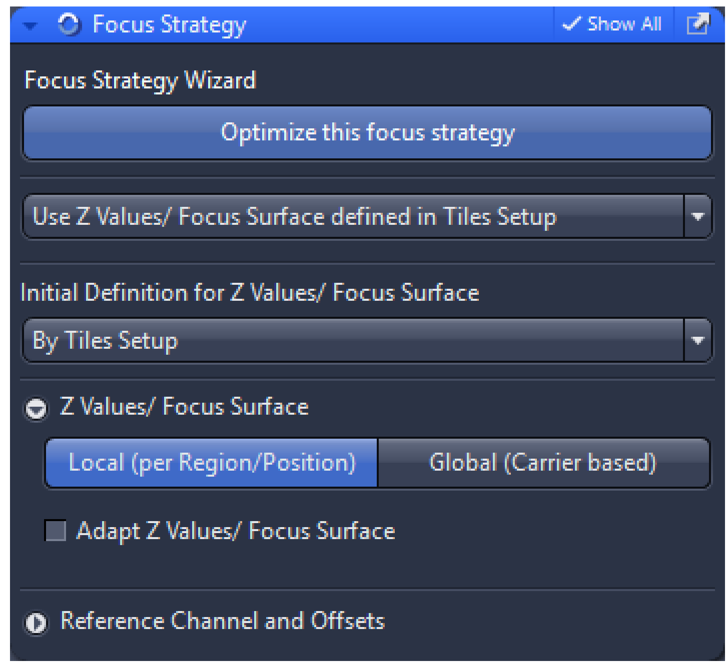
A local/global focus surface ensures that all tiles are in focus on tilted or irregular specimens. Local focus surfaces for tile regions are interpolated on the basis of the focus positions of support points. Positions automatically have a horizontal focus area with the Z-value of the position.

The following guide explains how to use the focus strategy for local focus surfaces.

Prerequisite

- ✓ To use the **Use Z Values/ Focus Surface defined in Tiles Setup** focus strategy, you need a licence for the **Tiles** module.
- ✓ You have read *Introduction [▸ 86]* for using focus strategies (experiment created, at least one channel defined, acquisition dimensions activated).
- ✓ You are on the **Acquisition** tab in the **Focus Strategy** tool.

1. Select the **Use Z Values/ Focus Surface defined in Tiles Setup** entry from the dropdown list (if not selected by default).
2. If you want to set up the entire focus strategy, you can click on Optimize this focus strategy to use the *Focus Strategy Wizard [▸ 716]*.
3. In the **Z Values/ Focus Surface** section select **Local (per Region/Position)**.
4. Under **Initial Definition for Z-Values/ Focus Surface** select the **By Tiles Setup** entry from the dropdown list.



→

5. Set up a **Tiles** experiment. For more information, see also the chapter *Tiles & Positions with Advanced Setup [▸ 515]*.
6. Create a **local focus surface** for the tile regions, see *Creating a Local Focus Surface [▸ 522]*.
7. To start the experiment, click on the **Start Experiment** button.

The tiles of tile regions and positions are acquired using the local focus surfaces.

If you are performing a **Time Series** experiment we recommend to use the **Adapt Z Values/ Focus Surface** functions, see *Focus Strategy Tool [▸ 711]*. By these you can correct the focus for small drifts that occur in long multidimensional time series experiments with living cells, for example.

9 Image Processing

9.1 Image Processing Workflow

On the **Processing** tab you can apply image processing functions (IP functions) to acquired or loaded images. The basic workflow is quite simple:

- Acquire or open an image that you want to process.
- Open the **Processing** tab.
  When you open the tab, the last active image (e.g from Acquisition tab) will be used as the input image for the processing function.
  If you want to select an other input load the new image under **Image Parameters | Input**. Note that the image must be opened in ZEN before.
- Select the desired processing mode (**Single / Batch**). Per default, single processing mode is selected. Batch processing allows to apply a specific processing function automatically to a batch of images.
- Select the desired processing function under **Method**, e.g. **Color Balance**.
  You can search for processing functions in the **Search** field. Therefore just enter the initials of the functions you want to search.
- Set the parameters of the function under **Parameters**.
  If you need help for a specific function and its parameters press the F1 key. You will find detailed descriptions for each functions in the online help.
- To see how the functions works you can click on the **Preview** button under **Image Parameters | Output**.
- Click on the **Apply** button to apply the processing functions to the image.
  This will create a new image in a new image container. The original image will not be changed.

See also

Processing Tab [▸ 664]

9.2 General Settings

Save/Load Settings

In the **Settings** section at the top of a function's **Parameters** tool you are able to save and reload the adjusted settings. If you have adjusted the parameters for a function, simply click on **Options > New** to save your setting under a new name.

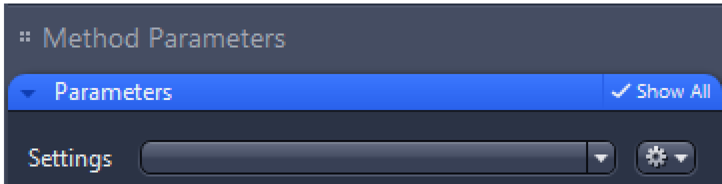


Fig. 10: Settings section

Adjust Settings per Channel

If your input image is a multichannel image, all settings can be adjusted individually for each channel. Therefore the checkbox **Adjust per Channel** must be activated.

Third Dimension Settings

If there are more dimensions in the input image and/or the **Show all** mode is activated the **Third Dimension** dropdown list becomes visible. There you can select to which dimension you want to apply the function additionally. Several choices are available, depending on your input image:

Parameter	Description
Third Dimension	Only visible, if there is a third dimension in the input image and/or **Show all** mode is activated. Here you can select how you want the function to work in the case of multidimensional images.
- 2D Slices	The function is used in a 2-dimensional fashion only for multidimensional images such as time lapse or z-stacks.
- Z, T or C	Here you can select to which additional dimension the functions should be applied to. If you select the **C** (channel) dimension, the **Adjust per Channel** checkbox is not available.
Adjust per Channel	Only available for images with multiple channels and if **C** is not selected as **Third Dimension**. **Activated:** Opens a list with the channels to allow an individual adjustment of each channel. For every channel you have the following options:
– Process Channel	Processes this channel according to the input.
– Skip Channel	Skips this channel when processing. This channel will not be in the output image.
– Copy Channel	This channel is copied into the output image without a reduction of noise.

Reset Settings to Default

You can reset the settings for a function to default by clicking on the **Defaults** button.

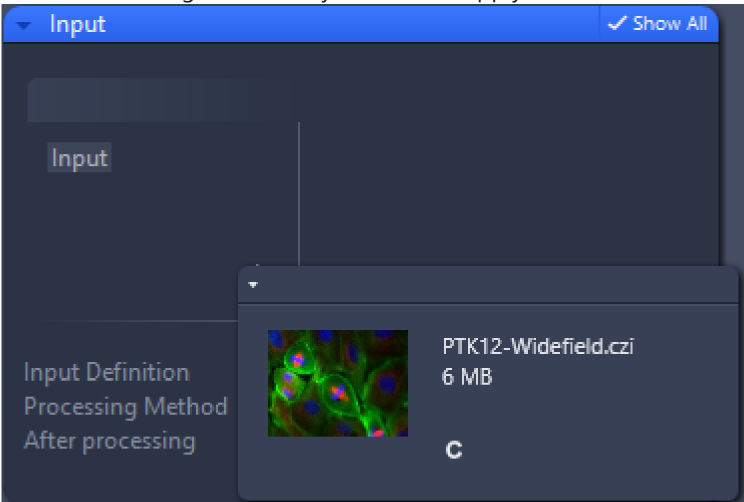
9.3 Performing Deconvolution Using Default Values

Successful deconvolution depends mainly on good image quality, knowledge about the optical parameters of the sample and detailed knowledge about the type of instrument used for image acquisition. While information about the used instrument type can be easily extracted from the image metadata, optical parameters of the sample might not be known and the image quality can vary widely. Many parameters are available for deconvolution which allow you to make corrections to the image quality and adjust the algorithms to match the various optical condiditions such as coverslip type or the medium, in which the sample is embedded. This wide range of parameters can be overwhelming.

With the **Deconvolution (defaults)** method, good initial result are achieved by using a carefully preselected set of default parameters. The parameters are automatically adapted to the following instrument types: widefield, confocal, lightsheet and ApoTome.
While these parameters usually give nice results, there are cases, where further parameter changes will be necessary, e.g., activating and using spherical aberration correction. In such cases, you should use the **Deconvolution (adjustable)** method.

Prerequisite
- ✓ You are on the **Processing** tab.
- ✓ You have acquired or opened a fluorescence image on which you wish to perform deconvolution.
- ✓ All tools are in **Show All** mode.

1. Under **Method Selection**, open the **Method** tool.
2. Click on the **Deconvolution** group.
   → You will see the methods **Deconvolution (defaults)** and **Deconvolution (adjustable)**.
3. Click on **Deconvolution (defaults).**.
4. Under **Method Parameters**, open the **Parameters** tool.
   → Here you will see 4 different algorithms for deconvolution (Nearest Neighbor, Inverse Filter, Fast Iterative, Iterative), which you can apply to your image automatically.
5. To select an algorithm, click on the relevant entry.
6. Under **Image Parameters**, open the **Input** tool.
   → You will see the **Input** tool. If the **Set Input Automatically** checkbox is checked, the currently active image has been loaded as input image automatically. If the checkbox is unchecked, the container for the input image is empty. In this case continue with step 7.
7. Click on the empty image container.
   → A list opens with preview images of all currently open images.
8. Click on the image to which you want to apply deconvolution.



   → The image can now be found in the image container and will be used as the input image for processing.
9. Click on the **Apply** button on the top to perform deconvolution, .

Deconvolution is performed. A new image file is generated and is opened automatically in the center screen area after processing. If you are satisfied with the result, save the processed image. Repeat deconvolution using the other default values to obtain different results. If you have expert knowledge, you can configure all the deconvolution settings yourself using the **Deconvolution (adjustable)** method.

9.4 Performing Configurable Deconvolution

These instructions explain how to deconvolve a Z-stack image correctly step by step.

We will use the best method **Constrained Iterative** and a **theoretical PSF**.

Preparation

To follow these instructions you will need a Z-stack image of your sample. You have opened the software and no images are loaded.

Overview

The following steps are described in these instructions.

Prerequisites

- You are on the **Processing** tab.
- You have acquired or opened a fluorescence image on which you wish to perform deconvolution.
- All tools are in **Show All** mode.

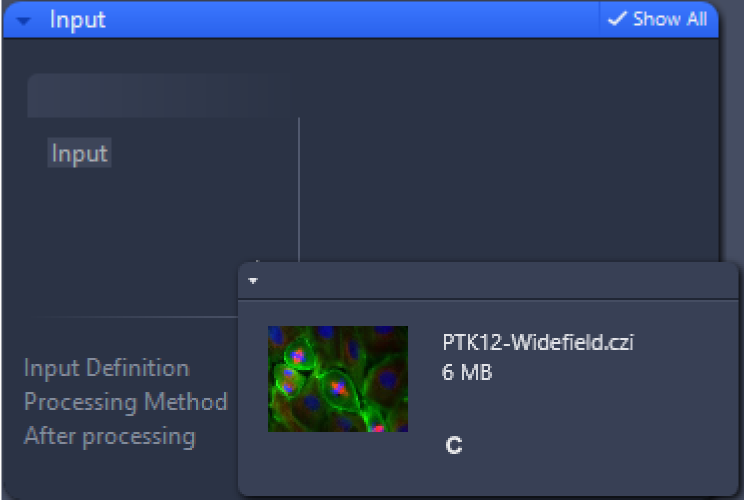
Steps

- **Step 1: Load input image**
  In this section you will find out how to load an input image in Deconvolution (Configurable).
- **Step 2: Set parameters**
- In this section you will find out how to set the parameters.
- **Step 3: Process image**
  In this section you will find out how to process the image and compare it with the input image.

9.4.1 Step 1: Load input image for deconvolution

In this step you will select the image to be processed and load it as an input image for deconvolution.

1. On the **Processing** tab **> Method** tool in the **Deconvolution** group select the **Deconvolution (adjustable)** method.
2. In the **Input** tool, select the image that you want to deconvolve. If the **Set Input Automatically** checkbox is checked, the currently active image has been loaded as input image automatically. Since this means, that the output image is then also automatically selected as input, it is recommended to uncheck this checkbox. Select the desired input image.



Info

If a warning appears at this point, it is likely that parameters required for deconvolution are missing from the image. You can subsequently enter or change these values in the **Parameters** tool > **PSF settings** tab > **Microscope** parameters.

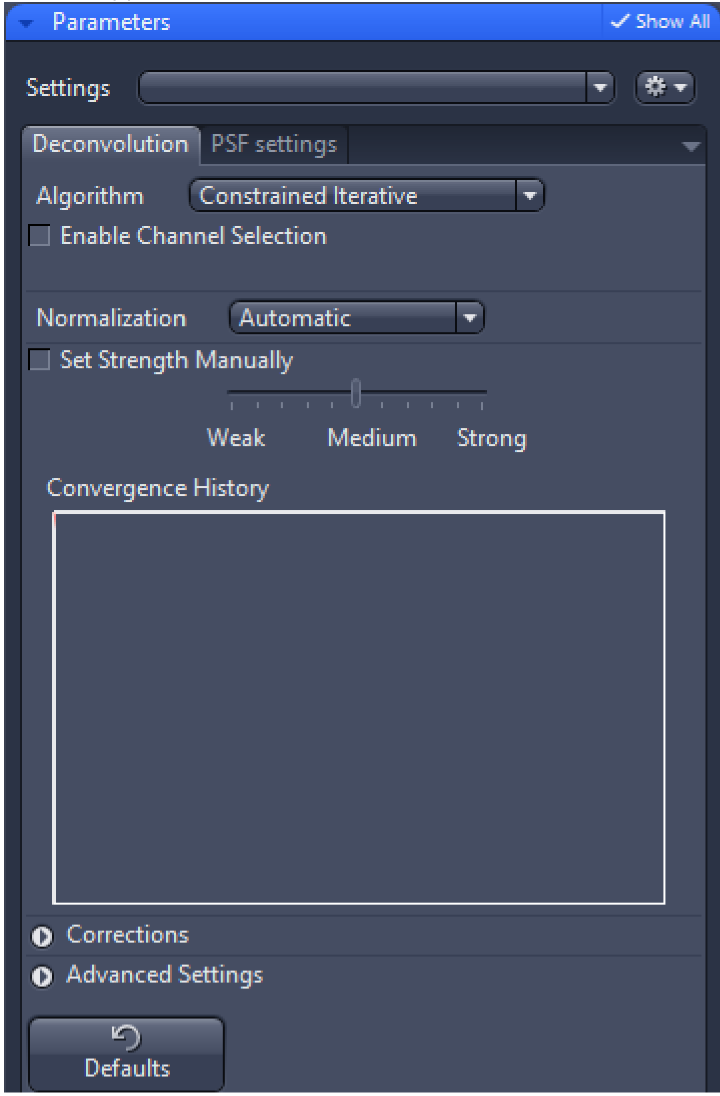
9.4.2 Step 2: Set parameters for deconvolution

In this step you will choose the desired algorithm and the associated method parameters. Then you will perform deconvolution and save the result.

Prerequisite ✓ On the **Processing** tab, in the **Method Parameters** group, you have opened the **Parameters** tool in the **Show All** mode. You can usually leave these parameters alone as they are automatically set to give you a good result.

1. On the **Deconvolution** tab first set the desired algorithm. In our example we are using the Constrained Iterative algorithm, which is the most complex algorithm, but normally the best one to use.

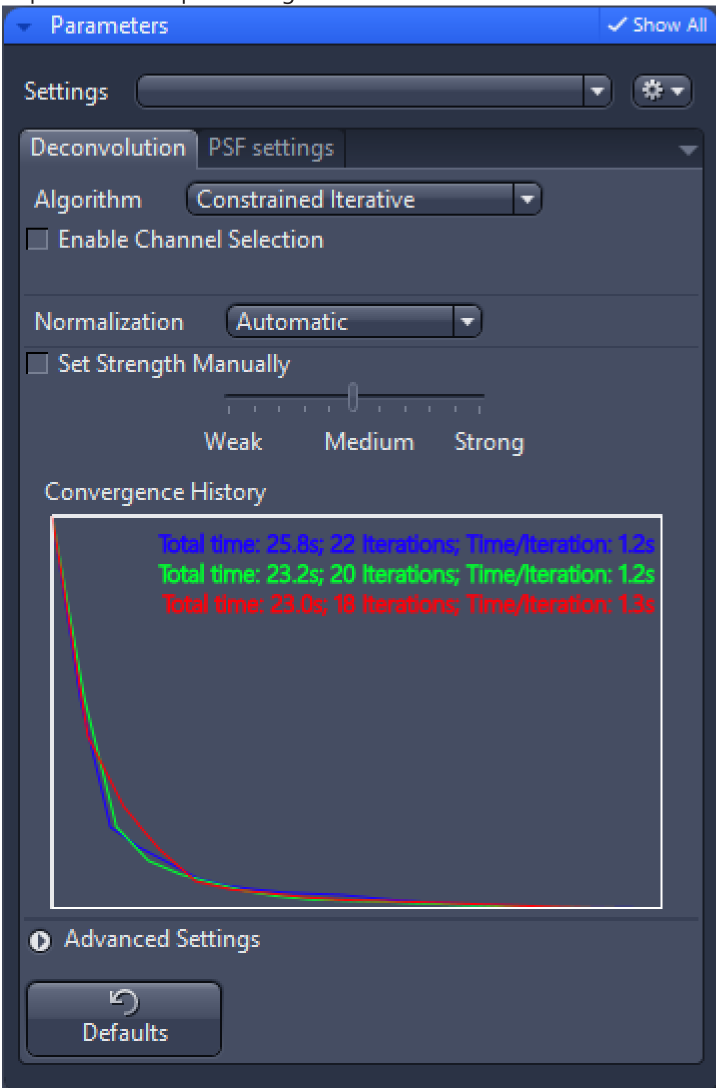
   → You will now see a number of additional parameters for the Constrained Iterative method appear on the tab (**Show All** mode).



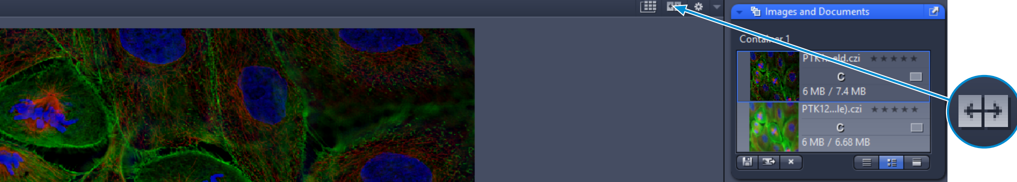
9.4.3 Step 3: Perform deconvolution

In the last step you will perform deconvolution. The resulting image will be compared with the input image and details relating to the processing procedure will be observed.

1. Go to the **Deconvolution** tab in the **Parameters** tool to keep the **Diagnosis** section in the foreground.
2. Click on the **Apply** button.
   → You can monitor the progress in the **Convergence History** graph, in which the gradual improvement is plotted against the number of iterations.



3. Save the resulting image under a meaningful name.
4. Click on the **Splitter Mode** button in the document bar. This creates a multi image (split image).



5. The currently active image is loaded automatically into the multi image. Now drag the input image from the document gallery in the Right Tool Area into the split document.

6. On the **Display** tab, change and adjust the display as desired, e.g. **Best Fit** with 0.01 and for black and white value with a **Gamma** of 0.8.

   → Both images are adjusted simultaneously.

7. If on the **Split Display** tab the **Synchronize Dimensions** checkbox is activated, you can now zoom synchronously into the images (mouse wheel) and, with the mouse wheel held down, move the image content as desired to focus on the regions of interest.

8. To create an image of the desired view click on **New Image from > Current View > Create**.

   → This creates a new output image document with both images shown side by side.

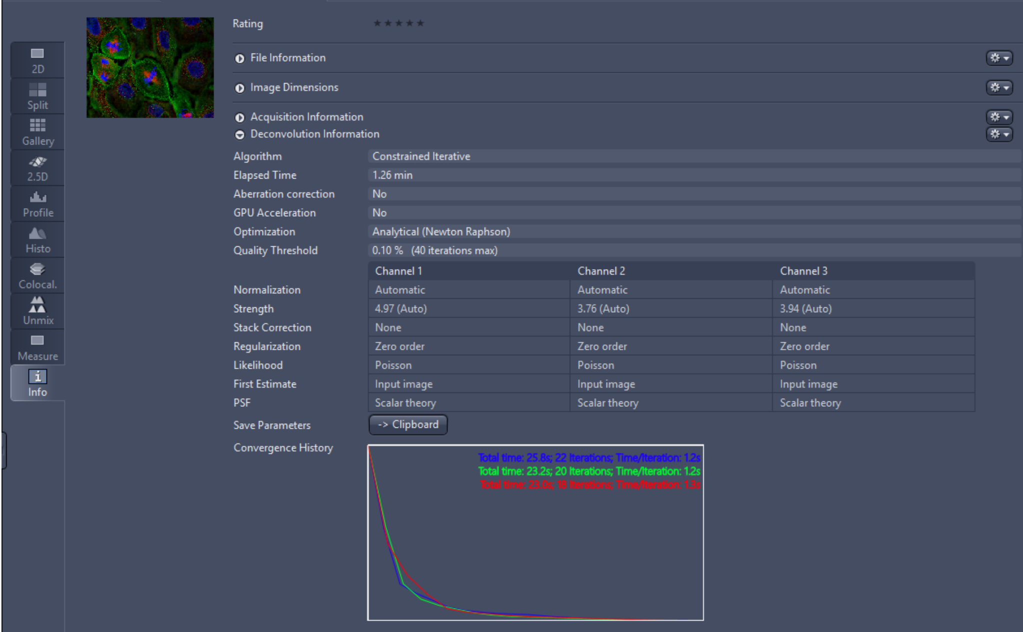
You have successfully performed deconvolution, observed the processing procedure and created an output image to compare the deconvolved with the input image.

9.4.4 Step 4: Info View and re-using Deconvolution parameters from a processed image

The **Info View** contains a section **Deconvolution Information**, which shows a summary of the parameters used for deconvolution of the image. It also contains the **Convergence History** graph displaying the time it took to get this image processed.

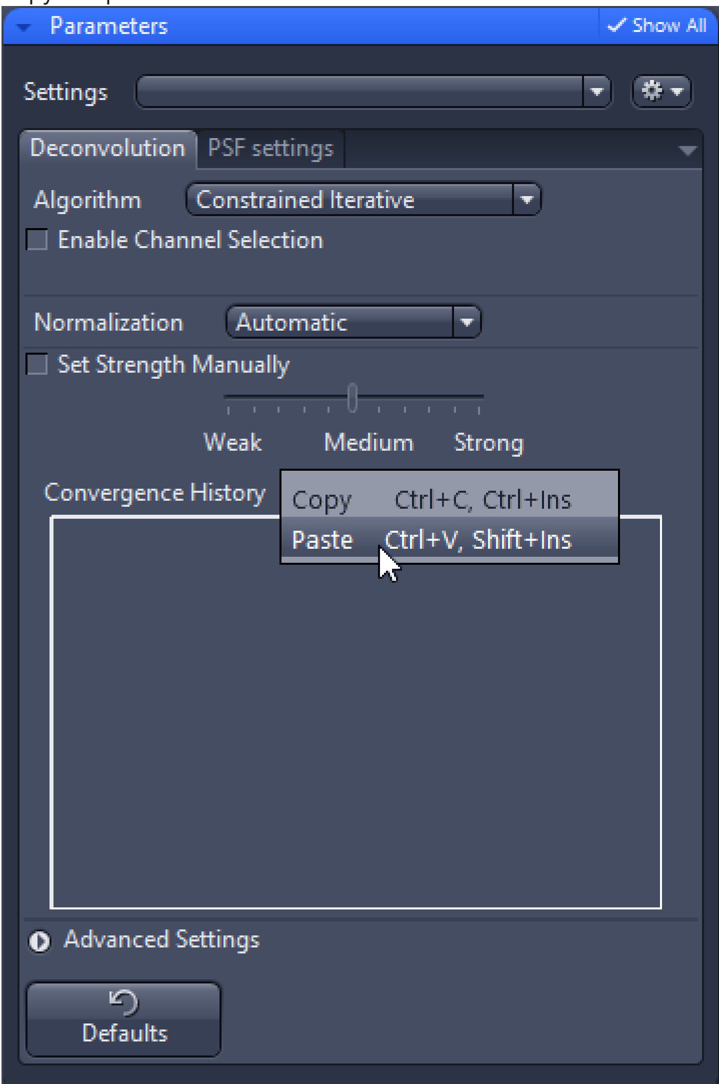
If you like to use the same settings in order to process another image, the following steps show how to do this:

1. Select the processed image from the previous steps.
2. Select the **Info View** and scroll down to the **Deconvolution Information** group:



3. Click on the **Clipboard** button.
   → All deconvolution parameters are copied to the clipboard.

4. Open the new image you want to deconvolve and select it as input in the **Deconvolution (adjustable)** function. Right-click and select **Copy** and then right-click and select **Paste** to copy the parameters into Deconvolution.



5. Click on the **Apply** button to run Deconvolution with the identical function parameters as used for the previous image.

9.5 Measuring the PSF using subresolution beads

When measuring the PSF (point spread function) you perform the following process. Note the prerequisite preparation and hints.

Preparation

1. The surface of coverslips is hydrophobic which means, liquid droplets do not spread out easily and beads tend to aggregate at the edges. For PSF measurements you want individually spread out beads.
2. Bath the coverslips for 10 minutes in 100% ethanol.
3. Use forceps to remove the coverslip. Shake off excess liquid and run through bunsen burner flame.
   → This makes the surface slightly hydrophilic which means, the droplets and beads spread out easier.

4. Break up agglomerates by sonicating stock suspension in a waterbath for 20 minutes. Stocks suspensions are way too dense, so dilute 1:100 with 70% ethanol.
5. Create further dilutions of 1:1.000 and 1:10.000 by adding 100 µl to 900µl 70% ethanol. Mix well using a Vortex mixer.
6. Put one 5 µl drop for each dilution on a cover slip using a 20 µl Eppendorf pipette
7. Let dry. This should take less than 5 minutes. You can speed it up by putting the cover slip on a warm surface.
8. Put 10-20 µl mounting medium on the coverslip. For aqueous mounting media seal edges of coverslip with valap (1:1:1 mixture of vaseline, lanolin, paraffin), nailpolish or paraffin.

In the next step, you acquire an image.

Imaging

1. Locate the beads on the microscope (e.g. use a 20 x lens first, then move to 63x oil). Start at the 1:100 spot which should have tons of beads and be easy to find.
2. When found, move to a sparser spot and try to find an area with a couple of single beads in the FOV. This can be tricky, but usually you will be able to find good areas if you keep looking around.
3. Acquire a Z-stack of a suitable area, observing the following rules.
   → No saturation. This can be tricky with LSM’s. Use 12-bit mode at least.
   → Only fill the dynamic range in the histogram to about 80%.
   → Make sure to focus up and down when setting up the exposure times to measure the exposure suitable for the bright bead center to avoid the risk of saturation.
4. Set up the z-stack as follows: in the Z-stack tool, click on the **Optimal** button.

   → This sets the distance according to Nyquist. For bead measurments further reduce the slice distance to about half of what **Optimal** suggests.
5. Also, define the top and bottom of the stack in such a way that the airy disc of the beads cannot be distinguished any longer.
6. When ready, save and name the image properly.

Look at the result in **OrthoView**: Do you see spherical aberrations? Are the beads symmetrical? Are there enough individual beads in the stack? Is the background low enough?

Processing in ZEN Blue

1. Use the PSF wizard (Processing/Deconvolution) to extract the PSF from the bead-z-stack. For more information, see *Creating a PSF - With Wizard and Without [▸ 135]*.

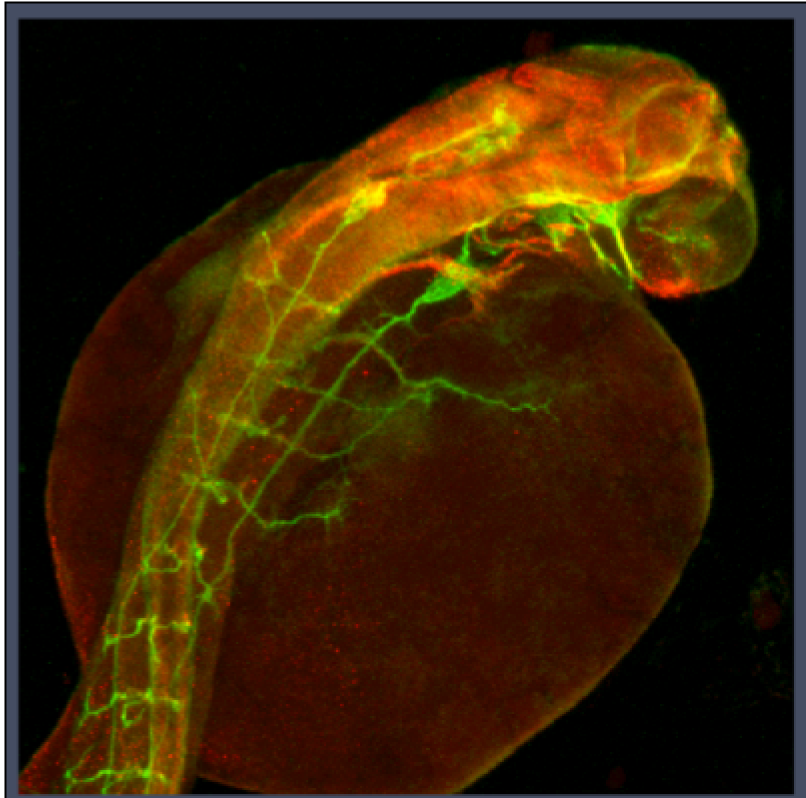
The PSF wizard guides you step by step through the necessary procedure.

The result of the wizard is a PSF file which you use in deconvolution for images acquired under the same conditions.

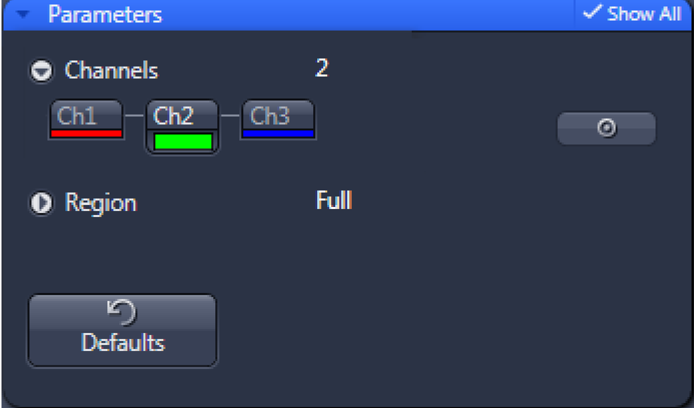
9.6 Extracting Individual Fluorescence Images of a Multichannel Image

In this topic we will show you how to extract individual fluorescence channel images of a multi-channel image.

Prerequisite ✓ You have acquired or opened a multichannel image.

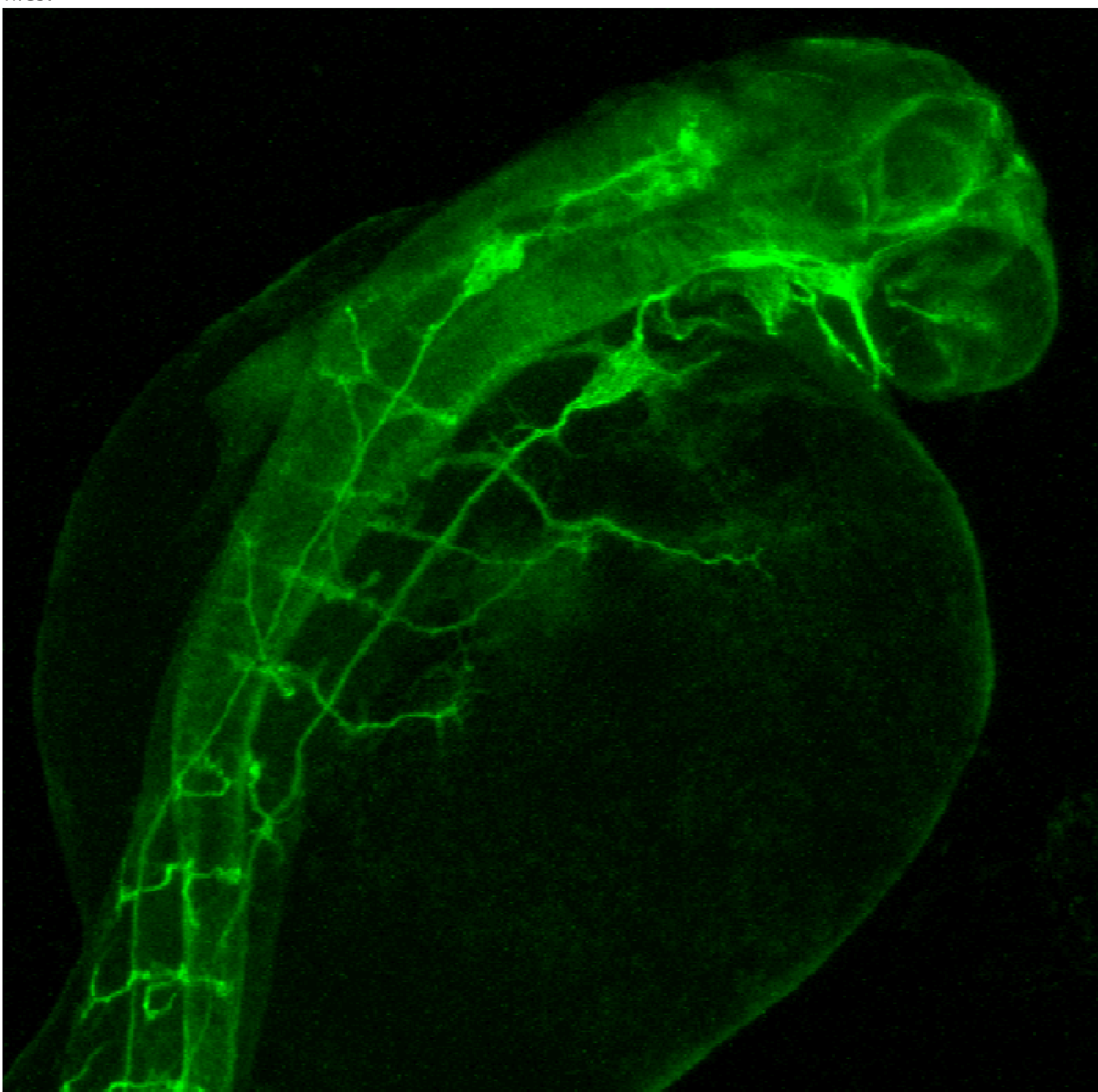


1. Select the **Processing** tab. Open the **Method** tool and select under **Utilities** the entry **Create Image Subset.**
2. In the section **Image Parameters** open the **Input** tool and select the multichannel image as Input image.
3. In the section **Method Parameters** open the **Parameter** tool and the select the entry **Channels**. Deactivate the channels you do not want in the extracted image (e.g. the red and the blue fluorescence channel).



4. Click on the **Apply** button.

In the example image, you have extracted the green fluorescence channel image of the multi-channel image. Activate the corresponding channel and deactivate the other channels to extract further individual fluorescence images. You can now save the extracted sub images as separate files.



9.7 Applying Batch Processing

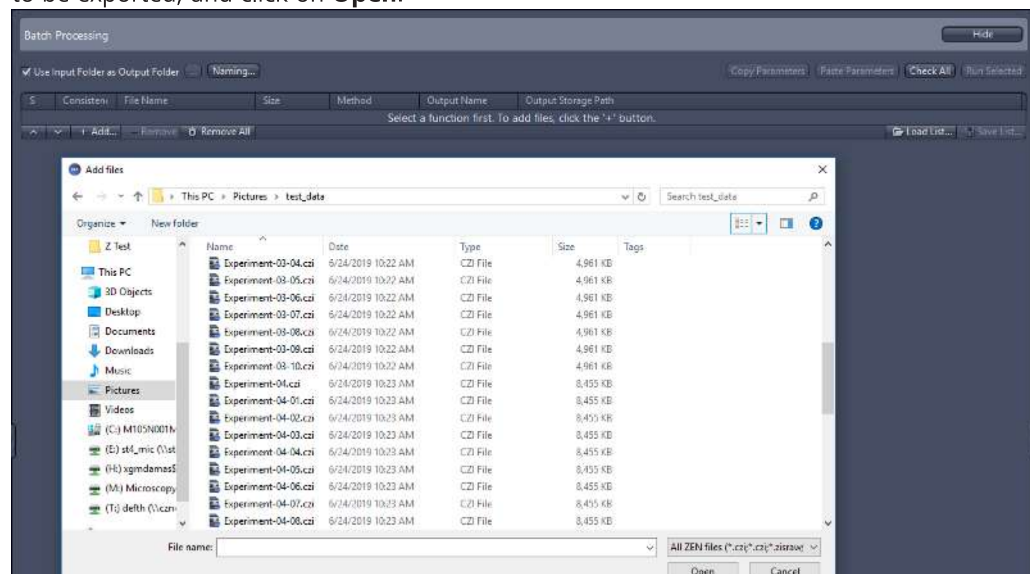
In this topic we will show you how to export all images of a folder as batch. For each image you can use identical or different export settings.

Prerequisite

✓ You have a folder with several CZI-images to be exported in a new image type (ie. TIFF, BMP, JPEG). For example two 2channel-Timelapse images, one 2channel-Z-stack image, one 3Channel image on the product DVD Example Image Databases\CaRationSeries.czi, kaede5lsm.czi, ratbrainstack.czi, zf_psa.czi).

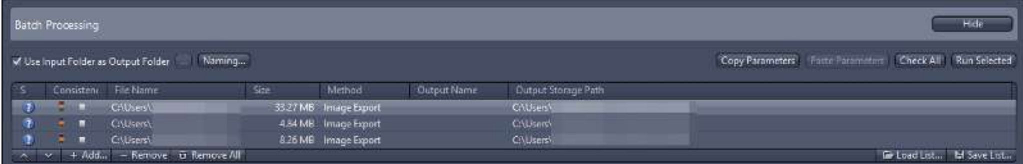
1. Select the **Processing** tab. Click on the **Batch** button.
2. Open the **Method** tool and select the entry **Image Export**.
3. In the **Method Parameters** section, open the **Parameters** tool.

4. In the **Batch Processing** tool, click on the **Add** button. Select the folder, mark all images to be exported, and click on **Open**.



→ The list of images is displayed in the **Batch Processing.**

5. Activate the checkbox **Use Input Folder as Output Folder** to save the exported images in the folder of the original images.
6. Click on one image in the list and do the export settings in the **Parameters** tool. In the example the TIFF format will be used for all time points and channels. This setting is only valid for the selected image, but can be copied to further images in the list with identical dimensions (i.e. 2channel Time-Series image).
7. Click on the **Copy Parameters** button. Select the image with identical dimensions in the list and click on the **Paste Parameters** button.
8. Continue with the other images of the list. Use **Copy Parameters** and **Paste Parameters** or define the export settings for each image individually in the **Parameters** tool.
9. Click on the **Check All** button. All images in the list will be tagged with a green marker, if they have the correct setting.



10. Click on the **Apply** button.

All exported images in the list will be checked and are exported in separate folders in the Input folder.

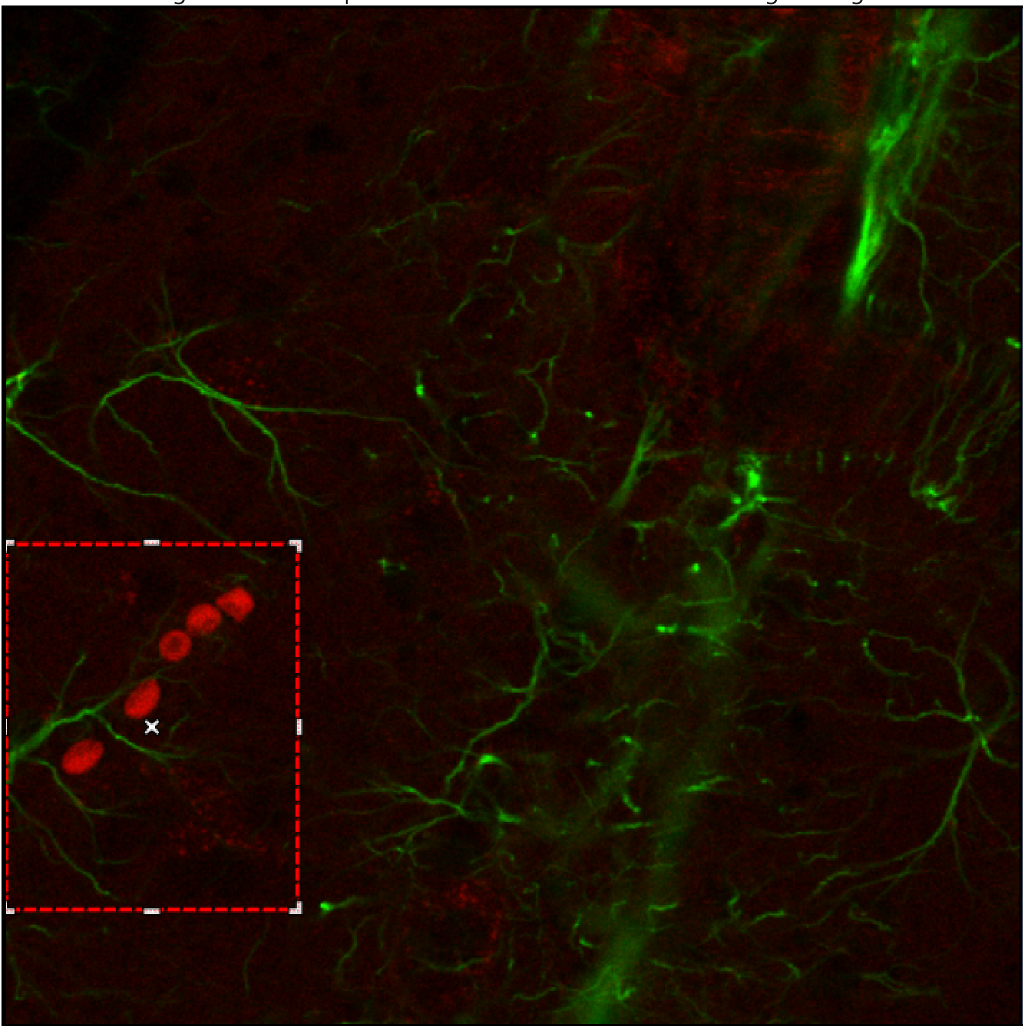
9.8 Cropping a ROI

In this topic we will show you how to crop a region of interest (ROI) of a image.

Prerequisite ✓ You have acquired or opened a multichannel image.

1. On the **Processing** tab, open the **Method** tool and under **Utilities** select the entry **Create Image Subset**.
2. In the **Image Parameters** section, open the **Input** tool and select the image.
3. In the **Method Parameters** section open the **Parameters** tool.
4. Open the **Z-position** dimension setting and select **Extract Range** of the dropdown list. Set the start position and the end position using the slider or the input field.

5. Open the **Region** setting and select **Rectangular Region** of the dropdown list.
6. Click in the image on the start position of the ROI and draw a rectangular region.



→ You have marked the ROI which should be cropped.

7. Click on the **Apply** button.

The marked ROI with the defined dimensions is extracted of the image. You can now save or process this image.

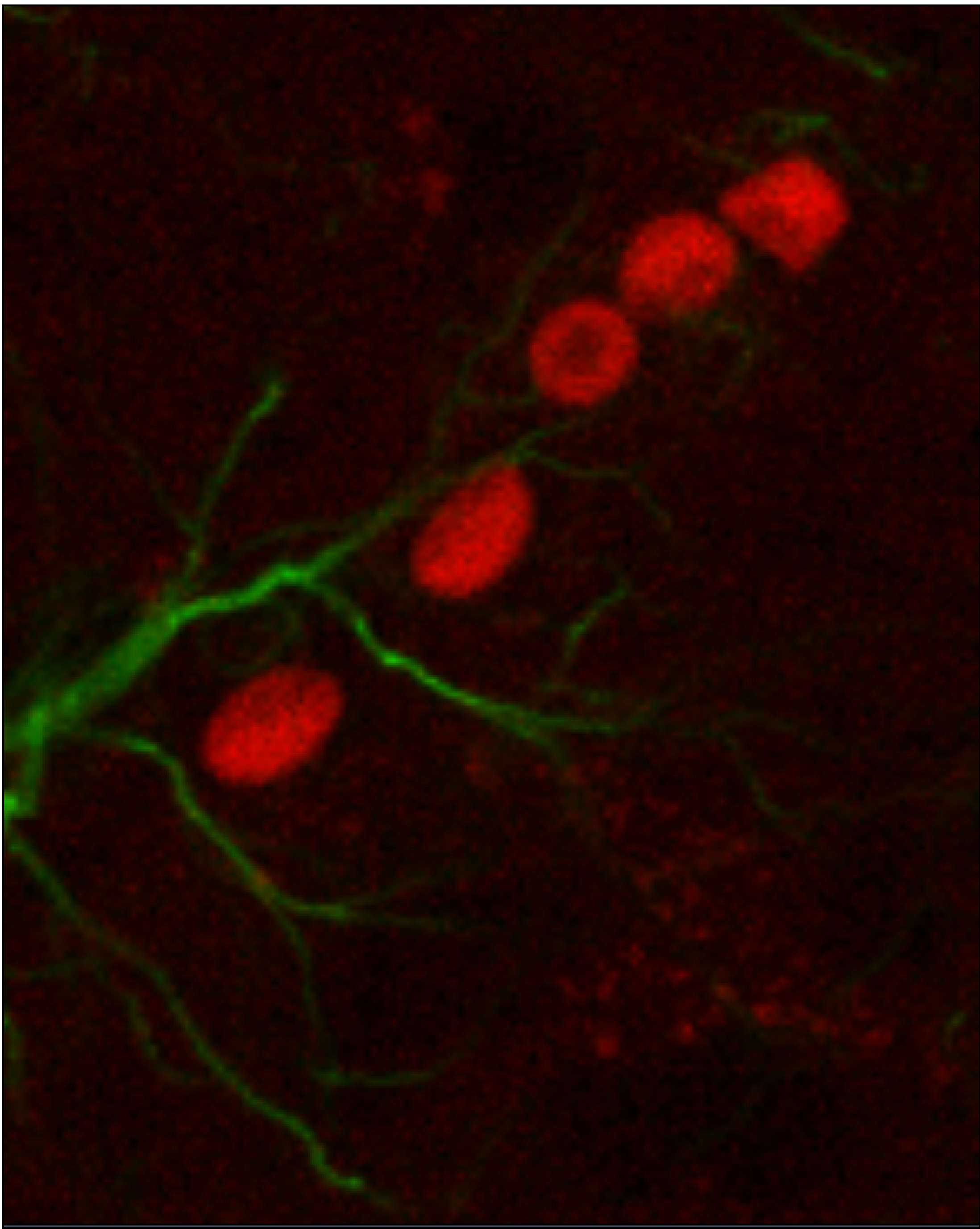


*Fig. 11: Cropped ROI with the dimensions **All channels** and the **Z-Postion 11-20**.*

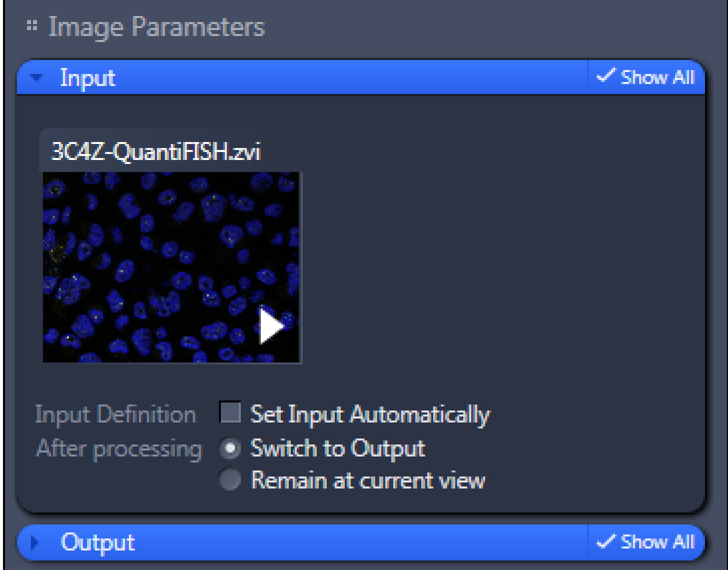
9.9 Creating an EDF Image

In this topic we will show you how to create an extended depth of focus (EDF) image of a Z-Stack image. The focus planes of all the z-positions will be calculated to one EDF image.

Prerequisite ✓ You have acquired or opened a z-stack image.

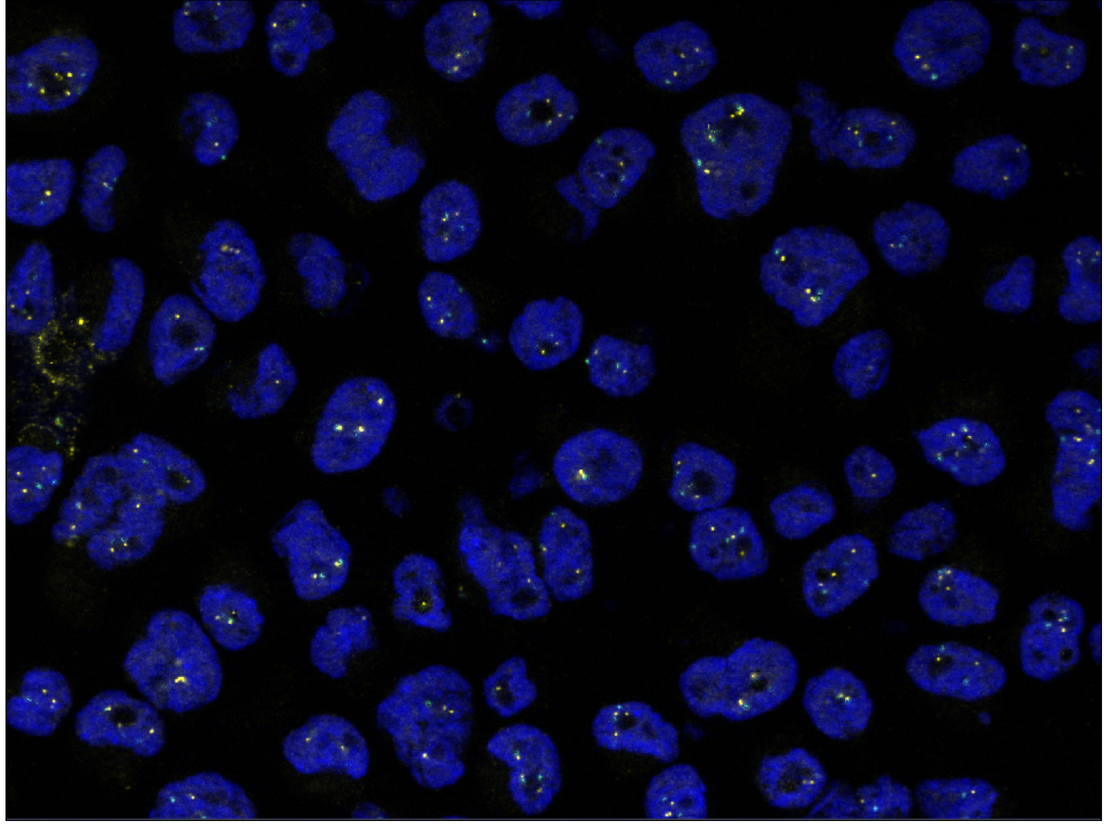
1. On the **Processing** tab, open the **Method** tool and select under **Sharpen** the entry **Extended Depth of Focus**.

2. In the **Image Parameters** section open the **Input** tool and select the Z-Stack image as input image.



3. In the **Method Parameters** section open the **Parameters** tool.
4. Select **Wavelets**of the **Method** dropdown list.
5. Select **High** of the **Z-Stack Alignment** dropdown list.
6. Click on the **Apply** button.

All focus planes of the Z-Stack image are now calculated to one EDF image.



9.10 Creating image subset and split dimensions

Depending on your loaded image, you can split certain dimensions, e.g., channels or timepoints, and save them to separate images. First, you select the method, and in the **Parameters** tools, you specify the dimension. Each of the dimensions is only visible if the corresponding dimension is present in the input image.

Create Image Subset and Split opens the resulting images in the ZEN document area. Therefore, for this image processing function, Split Dimension is limited to a maximum of 20, e.g. timepoints, tiles, or slices. To split images along a dimension with more than 20 elements, use **Create Image Subset and Split (Write files)**, which creates image files in the specified folder.

Prerequisite ✓ You have loaded an image with more than one channel and not more than 20 timepoints.

1. Select **Processing** tab **|** **Method** tool, and select under **Utilities** the entry **Create Image Subset and Split** or **Create Image Subset and Split (Write files)**. When using the first option, you can specify the output in the **Output** tool.
2. To create a subset from the available dimensions, make your settings in the sections **Channels** and **Time**.
3. In the **Parameters** tool, in the **Split Dimension** section, select the dimension along which you want to split the image, e.g., **Channel** or **Time**.
   In the **Channels** list, all channels of the loaded image are listed. Unselect channels you want to exclude.
   In the **Time** section, select the subset of timepoints which you want to include. If you have an image with more than 20 timepoints, and you limit the range to below 20 elements, **Time** will appear in **Split Dimension** allowing you to split the dataset along this dimension (for **Create Image Subset and Split**).
   In the **Region** section, select the region which you want to extract from the image, e.g., **Full** or **Rectangle Region**.
   Select **Rectangle Region** and draw a rectangle in the 2D view to specify the region you want to extract.
   When you use **Create Image subset and Split (Wirte files)**, you also need to specify the output folder for the images.
4. In the **Input** tool, specify whether you want to set the input automatically and which image you want to display after processing. Check the according options.
5. In the **Output** tool, specify how to proceed with the new image. Select if you want to overwrite an existing image with the same name or if you want to create a new output. The output tool is only available, if you selected the **Create Image Subset and Split** method.
6. Click the **Apply** button.

The new images are created according to your specifications.

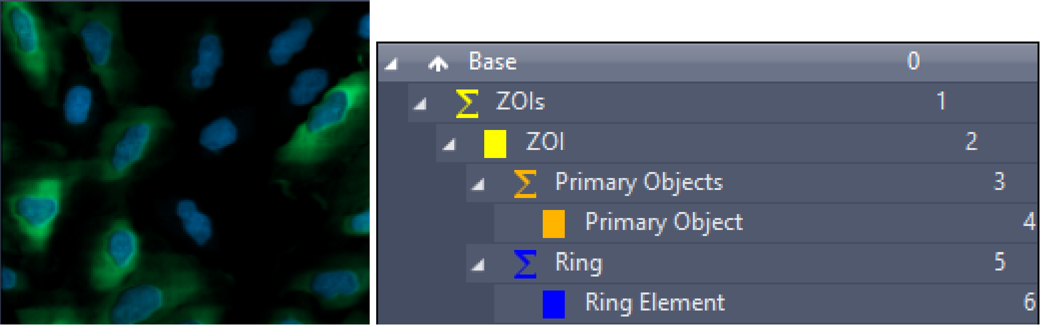
For more information, see *Create Image Subset and Split [▸ 186]* or *Create Image Subset and Split (Write files) [▸ 187]*.

9.11 Using Analyze to Label Image

The image processing functions **Analyze to Label Image** and **Analyze to Label Image Batch** label images based on an existing image analysis setting and the parameters which you select from the function. For general information about the image processing, see *Image Processing Workflow [▸ 90]*. For a description of all the parameters of **Analyze to Label Image** see *Analyze to Label Image [▸ 214]* and for **Analyze to Label Image Batch** see *Analyze to Label Image Batch [▸ 215]*.

The image analysis with this functions offers a wide variety of parameter options and the outcome completely depends on the combination of them. Therefore, this chapter can only give an example to illustrate the use and outcome of the function. In general, the labeling is possible as regions, contours, and double contours. For all three the same options are given in **Label Mode**.

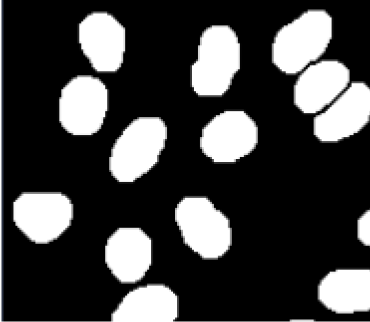
As an example, the following two channel image and image analysis setting with three classes are used to illustrate the functions:



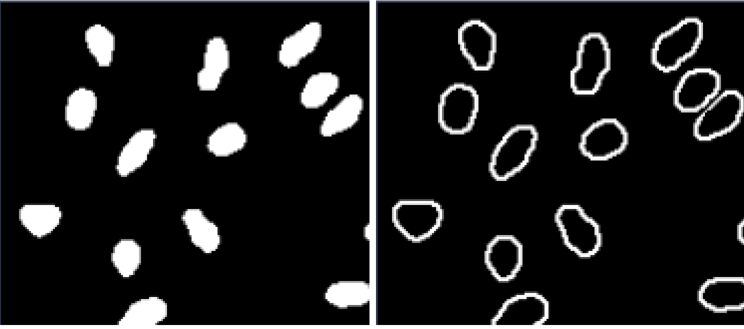
Example: Applying One channel and One channel per class

With these options, you can initialize your output image either as an image with one labeled channel or as an image where the labels for each class have a separate channel.

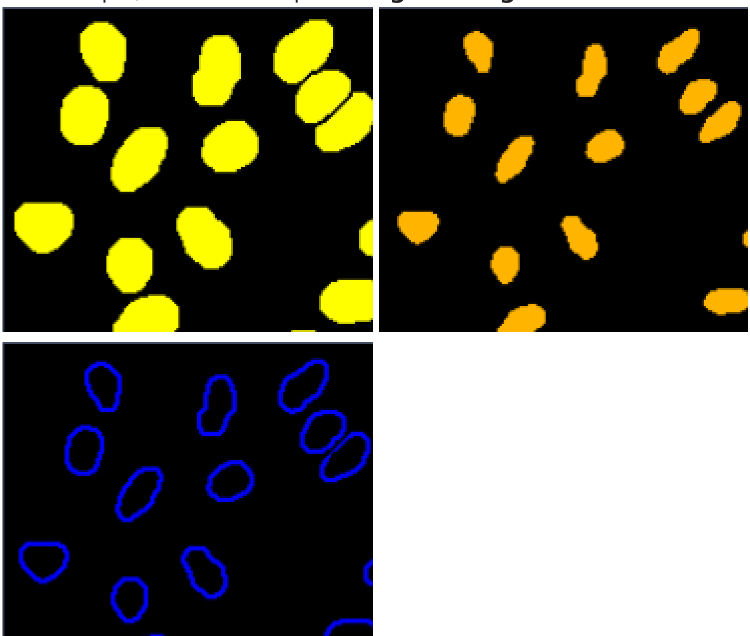
Labeling regions with the maximum pixel type as a 8 bit black and white image generates the following output for the first class:



Labelling the other two classes with the option **One channel per class** additionally creates the output for the other two classes:



If you want the regions to have the same color as defined in the image analysis setting, you can, for example, choose the option **Region - Region Class Color** and **24 Bit RGB** as **Pixel Type**:



As stated before, you can choose the same options (labeling with pixel type maximum and region class color) for contours or double contours as well.

9.12 Image Processing Functions

9.12.1 EM Processing

This group lists image processing functions that are designed for processing FIB-SEM images. Some of the functions can also be found in other groups of image processing functions. This group is only visible if you have the license for **EM Processing Toolbox**.

See also

EM Processing Toolbox [355]

9.12.1.1 Change Pixel Type

This method allows you to change the pixel type of an image. This can be useful if you want to compare or combine images that have different pixel types.

Parameters

Parameter	Description
Pixel Format	Select the desired pixel format from the dropdown list.
- 8 Bit B/W	The output image is a monochrome image, the whole-number gray values of which can lie in the range from 0 to 255.
- 16 Bit B/W	The output image is a monochrome image, the whole-number gray values of which can lie in the range from 0 to 65535.

Parameter	Description
- 32 Bit B/W Float	The output image is a monochrome image with real numbers as pixel values.
- 2x32 Bit Complex	The output image is a monochrome image with complex numbers (real part and imaginary part) as pixel values. Such images are generally created by means of transformation into the Fourier space.
- 24 Bit RGB	The output image is a color image, the whole-number color values of which in the red, green and blue channels can lie in the range from 0 to 255.
- 48 Bit RGB	The output image is a color image, the whole-number color values of which in the red, green and blue channels can lie in the range from 0 to 65535.
- 2x32 Bit RGB Float	The output image is a color image with real numbers as color values in the red, green and blue channels.
- 3x64 Bit RGB Complex	The output image is a color image with complex numbers (real part and imaginary part) in the red, green and blue channels. Such images are generally created by means of transformation into the Fourier space.

9.12.1.2 Coarse Z-Stack Alignment

This function enables you to align planes of z-stacks manually.

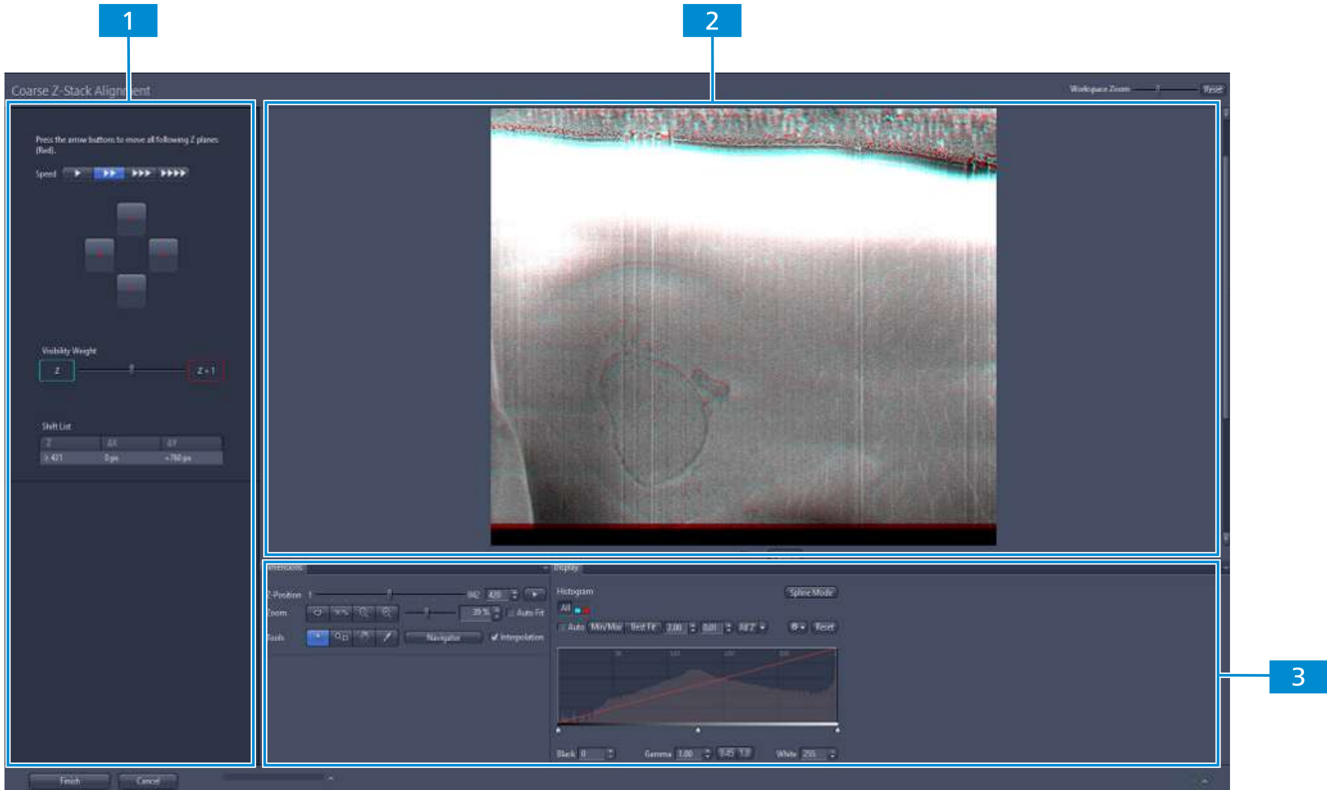
Parameter	Description
Z-Alignment Setup	The **Setup** button opens the **Coarse Z-Stack Alignment** setup to manually align your z-stack.
Shift List	Displays a list with all the shifts that have been made in the **Coarse Z-Stack Alignment** setup.

See also

Aligning z-planes manually [357]

9.12.1.2.1 Coarse Z-Stack Alignment Setup

With this setup you can manually align the planes of a z-stack. If you have imported your z-stack and there are bigger shifts between individual planes of the stack, this setup helps you to make a coarse alignment of your z-stack before an automated alignment, e.g. via **Z-Stack Alignment with ROI**.



1 Alignment Controls Section
Here you have your controls to align the z-planes in the stack manually. For more information, see *Alignment Controls Section [▸ 111]*.

2 Image View
Displays your selected z-plane and the following plane with different colors. The current plane is displayed in cyan and the following z-plane is displayed in red.

3 View Options
Here you have your standard view options (*Dimensions Tab [▸ 887]* and *Display Tab [▸ 902]*).

See also

Aligning z-planes manually [▸ 357]

9.12.1.2.1.1 Alignment Controls Section

This section of the Coarse Z-Stack Alignment setup contains the main controls to align the z-planes of your stack manually.

Parameter	Description
Speed	Selects the speed/ number of pixels by which the z-planes are shifted per click.

Parameter	Description
– ▶	Shifts the z-planes by 1 pixel per click.
– ▶▶	Shifts the z-planes by 10 pixels per click.
– ▶▶▶	Shifts the z-planes by 100 pixels per click.
– ▶▶▶▶	Shifts the z-planes by 1000 pixels per click.
Arrow Buttons	Shifts the next and all following z-planes in the corresponding direction.
Visibility Weight slider	Changes the color intensity of the current plane and the following z-planes in the **Image View**. In extreme slider positions, only the current z-plane or only the following z-planes are displayed.
Channels	Only available for images with multiple channels. Selects the channel that is displayed in the **Image View**. The same shift is applied to all channels of the image.
Shift List	Displays a list with all the shifts in x and y direction that you defined in the setup.

9.12.1.3 Create Image Subset

This method allows you to extract parts from one image and use these to create a new image. You can select these parts freely from the individual dimensions of the image.

Info

Each of the sections described below is only visible if the corresponding dimension is present in the input image.

Parameter	Description
Channels	Here you can select which channels of the input image you want to be used. All channels are selected by default. To deselect a channel, click on the respective channel button.
Z-Position, **Time**, **Block**, **Scene**	Here you can select which parts of the input image you want to use for the resulting image.
- Extract All	If selected, all parts of the corresponding image are extracted.
- Extract Single	If selected, you can select a single image to be extracted.
- Extract Range	If selected, you can select a certain range of images to be extracted.

Parameter	Description
- Extract Multiple	If selected, you can select several continuous ranges and individual sections. Enter one or more sections that you want to select in the input field. To do this, enter the first section, followed by a minus sign, and then the last section. If you want to define an interval, after the last section enter a colon and then the interval. The entry "2-10:2" means that every second section is selected from section 2 to section 10. Enter a comma after the first section if you want to define another section. You can also select individual sections separated by commas. By entering "2-10:2,14-18,20,23", you select every second section from section 2 to section 10, followed by sections 14 to 18, as well as sections 20 and 23.
- Get current position	Adopts the position from the current display in the image area.
- Interval	**Activated:** Interval mode is active. The Interval spin box/input field appears. Enter the desired interval here. E.g. if you enter the value 2 only every 2nd value from the range is considered.
Region	Here you can select if you want to use the entire image or just a region (ROI) of the input image.
- Full	Select this option to use the full image for the new image.
- Rectangle region (ROI)	Select this option to draw in a rectangle region of interest which will be used for creating a new image. If a rectangle region was drawn in you can see and change its coordinates by editing the **X/Y/W/H** input fields.
- Keep tiles	Has only an effect, if a region (ROI) is defined. **Activated:** Extracts the drawn in region including the complete tiles. This setting is recommend when you want to apply DCV processing functions on the resulting image.

9.12.1.4 Cut Out Regions

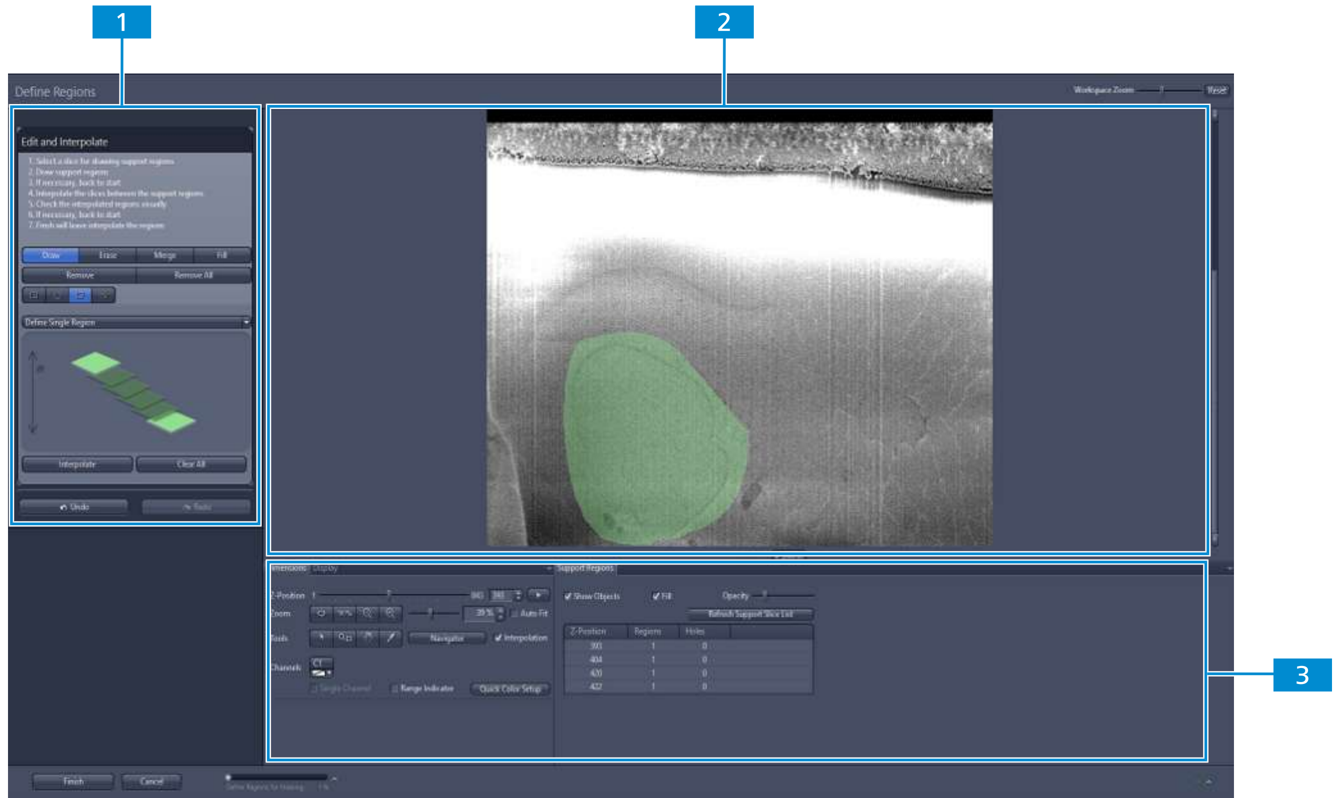
This function allows you to define a region of interest in your z-stack and cut it out of the stack as a separate volume.

Parameter	Description
Define Regions	Opens the *Define Regions setup [▸ 114]* to define the region of interest to be cut out of your z-stack.

See also

Cutting out a volume from a z-stack [▸ 359]

9.12.1.4.1 Define Regions setup



1 **Region Edit Section**
Here you have the controls to draw and edit the support regions. For detailed information see *Region Edit section [▸ 114]*.

2 **Image View**
Here you can draw your support regions into the current z-plane and edit them if needed.

3 **View Options**
Here you have some standard view options as well as the *Support Regions tab [▸ 115]*.

See also

Cutting out a volume from a z-stack [▸ 359]

9.12.1.4.1.1 Region Edit section

With this tools you can draw regions into your image and edit them.

Parameter	Description
Draw	Draws a new region into the image.
Erase	Using this button you can erase parts of a region. Holding down the left mouse button, outline the parts of the region that you want to erase. Right-click to erase the parts.
Merge	Use this button to connect regions. Holding down the left mouse button to extend the outline of an existing region or draw a connection between the regions that you want to merge. Right-click to merge them.

Parameter	Description
Fill	Fills a hole. To fill a hole, left-click on the hole.
Remove	Removes a region by clicking on it.
Remove All	Removes all regions on the current z-slice.
Rectangle	Enables you to add a rectangular region.
Circle	Enables you to add a circular region.
Contour	Enables you to add a polygonal region.
Contour (Spline)	Enables you to add a polygonal spline region.
– Define Single Region	In this mode you should only define a single region on each support slice of the image. For the interpolation, the support regions on the different slices do not necessarily have to overlap with one another.
– Define Multiple Regions	In this mode you can define multiple regions on each support slice of the image. Interpolation here is only possible, if the support regions on each slice have some overlap with the respective regions on the previous/next support slice.
Interpolate	Interpolates regions in the slices between the drawn support regions and displays the interpolated regions in the image.
Clear All	Removes all regions (drawn support regions and interpolated ones) in the entire stack.
Undo	Undoes the last change made on the current slice.
Redo	Restores the last undone change on the current slice.

See also

Cutting out a volume from a z-stack [▹ 359]

9.12.1.4.1.2 Support Regions tab

Parameter	Description
Show Objects	**Activated:** Displays the regions in the Image View. **Deactivated:** Hides the regions in the Image View.
Fill	**Activated:** The region is filled with color. **Deactivated:** Displays only the outlines of the regions.
Opacity	Sets the opacity for the regions.
Refresh Support Slice List	Updates the list below with the drawn support regions.

Parameter	Description
Support Slice List	Displays a list with information on the **Position(s)** where support regions have been drawn and the number of regions and holes on the respective slice. This list is only updated when you click on **Refresh Support Slice List**.

9.12.1.5 Denoise

This method removes noise from images using a real or a complex wavelet transformations. The process of denoising an image can be broken down into the following three parts:

- Calculate the wavelet transform of the noisy image.
  The wavelet transformation can be calculated by the method **Real Wavelets** and **Complex Wavelets**.
- Modify the noisy wavelet coefficients.
  This is done by using bivariate shrinkage with local variance estimation (thresholding). [Bivariate Shrinkage with Local Variance Estimator, Levent Sendur and Ivan W. Selesnick, IEEE Signal Processing Letters, Vol. 9, No. 12, December 2002]
- Compute the inverse transform using the thresholded coefficients.

Parameter	Description
Method	
- Complex wavelets	The Dual Tree Complex Wavelet transform provides better results due to the fact that it is nearly direction invariant and makes more directional sub bands available. The results will be less prone to block-artefacts. However, this method is computationally more intense and therefore takes longer.
- Real wavelets	The real wavelet transform only considers three sides (XYZ) and is therefore faster. However, the result can show block artefacts.
Strength	Here you adjust the strength with which the function is applied.

See also

Using Direct Processing [▶ 346]

9.12.1.6 Enhance Local Contrast

With this function you can improve the contrast of your image.

Parameter	Description
Clip Limit (%)	Defines the value at which the histogram is clipped. It is used to avoid oversaturation of the image in homogeneous areas. It is determined by the normalized histogram of the local region. The higher the value is set, the higher the contrast will be.
Region Size (%)	Defines the size of every single region or tile where the local histogram is calculated as percentage of the image size. For example, a region size of 50% has the size of half the image. A small region size will increase the contrast but will take more calculation time.

Parameter	Description
Bins	Defines the number of histogram bins that is used to create the contrast enhancing transformation. A higher number of bins will cover a greater dynamic range but will increase calculation time.
Process Tiles Separately	Only visible for tile images. **Activated:** Processes each tile region separately.

NOTICE

Difference between Result and Preview

The actual result of the function and the preview you see in the preview window can be different. This is caused by the parameter **Region Size (%)** and the different size of the preview and original image. The function splits the image into small regions and processes them individually based on the histogram of every single region. The histogram is usually related to the absolute region size. For the preview only the visible part of the image in the view is processed.

9.12.1.7 Gauss

This method allows you to reduce noise in an image. Each pixel is replaced by a weighted average of its neighbors. The neighboring pixels are weighted in accordance with a two-dimensional Gauss bell curve.

Parameter	Description
Sigma	Here you can adjust the sigma value. If the **Show All** mode is activated you can adjust the values in each dimension individually.

9.12.1.8 Highpass

This method performs high-pass filtering. The high pass filter is defined as the difference between the original image and the low-pass filtered original.

Parameter	Description
Normalization	Depending on the image processing function you have selected not all choices are available in the list.
- Clip	Gray levels that exceed or fall below the specified gray value range are automatically set to the lowest/highest gray value (black or white). The effect corresponds to underexposure or overexposure. This means that in some cases information is lost.
- Automatic	Automatic normalization of gray values to the available gray value range.
- Wrap	If the result is greater than the maximum gray value of the image, the value maximum gray value +1 is subtracted from it.
- Shift	Normalizes the output to the value gray value + max. gray value/2.
- Absolute	Values are used positive only.

Parameter	Description
Count	Here you set the number of repetitions. I.e. the number of times the function is applied sequentially to the respective result of the filtering. The effect is increased correspondingly.
Kernel Size	You can set the filter size in the x-, y-and z-direction, symmetrically around the subject pixel. This should be the size of the transition region between objects and background match.

9.12.1.9 Import SmartFIB TIFFs

This function allows you to import SmartFIB stacks of Crossbeam microscopes into ZEN.

Parameter	Description
Select Files	Opens a file browser to select the images for import.
Z-Spacing	Sets the slice distance in z. Per default, **Auto** is activated and the distance is calculated automatically with information from the image metadata of the first and last selected image. Deactivate **Auto** to enter a value manually.
XY-Scaling	Sets the scaling in x and y. Per default, **Auto** is activated and the scaling is automatically taken from the image metadata of the first selected image. Deactivate **Auto** to enter a value manually, e.g. if you import images without scaling information in the metadata.
Sample Angle	Sets the angle of the sample. Per default, **Auto** is activated and the sample angle is automatically calculated from the image metadata or set to the default of 54 degree and the image is rendered with a 90 degree tilt (if no information is available in the metadata). In this case the SmartFIB stack will be displayed perpendicular to the images acquired on a light microscope. Deactivate **Auto** to enter a value manually, e.g. if you know the original sample angle as set in the SmartFIB software and import images without using the information in the metadata.
Read XY Offsets	**Activated:** Reads the xy offset of the individual slices from the metadata. Note that this can lead to a slanted z-stack depending on the sample orientation during imaging and the metadata.

9.12.1.10 Median

This method allows you to reduce noise in an image. Each pixel is replaced by the median of its neighbors. The size of the area of the neighboring pixels considered is defined by a quadratic filter matrix. The modified pixel is the central pixel of the filter matrix. The median is the middle value of the gray values of the pixel and its neighbors sorted in ascending order.

Parameter	Description
Kernel Size	Here you can adjust the size of the filter matrix. If the **Show All** mode is activated you can adjust the values in X ,Y and Z direction individually.

9.12.1.11 Not

This function performs a binary "not" operation on all bits of the binary representation of an input pixel's gray value. A 0-bit in the input pixel results in an 1-bit in the corresponding output pixel an 1-bit in the input gets a 0-bit in the output. For integral image types the resulting output gray value is the difference of the maximum possible gray value minus the input gray value, but for float image type the results are strange due to the inhomogeneous float format.

9.12.1.12 Remove Stripes

This function allows you to remove stripe artifacts from your image. It is recommended to use the output preview to determine the suitable values for your parameters. You can display the output preview window by clicking on **Preview** in the *Output tool [769]*.

Parameter	Description
Method	Selects the method you want to use for the removing the striping artifacts in your image.
– GPU VSNR	Only available if you have a NVIDIA GPU in your machine. Uses a special GPU-based VSNR algorithm.
– GPU	Only available if you have a NVIDIA GPU in your machine. Uses a GPU optimized algorithm to remove the stripe artifacts from the image. This calculation produces the same results as the CPU version when using the same parameter values.
– CPU	Uses a CPU optimized algorithm to remove artifacts from the image.
Iterations	Sets the number of iterations after which the calculation is ended.
First Filter	Displays the settings of the first filter to remove vertical or horizontal stripes in your image.
– Noise Level	Sets the intensity level of the stripes in your image.
– Width	The calculation for the removal of stripes in the image is based on a Gaussian curve. This parameter sets the width of the curve.
– Height	The calculation for the removal of stripes in the image is based on a Gaussian curve. This parameter sets the elongation of the curve.
– Angle	Sets the angle for stripes in your image. An angle of 0 corresponds to vertical stripes. An angle of 90 (or -90) corresponds to horizontal stripes.
Second Filter	To remove a second set of stripes (e.g. if you have both vertical and horizontal stripes in your image), you can activate a second filter. For information about the parameters, see the descriptions above.
– Enabled	**Activated:** Displays the settings for a second filter to remove striping artifacts in the image. **Deactivated:** Hides the settings for a second filter and only the first filter is applied.

9.12.1.13 Shading Correction

This method allows you to improve images in which the quality has been impaired by uneven illumination or vignetting.

If you want to perform shading correction before an experiment (recommended) you have to use the shading correction function in the **Camera** tool in the *Post-Processing section [▸ 673]*.

Parameter	Description
Shading Mode	
- Camera shading	Applies the Shading correction to each tile of a tile image.
- Global shading	Applies the Shading correction to the whole tile image. Requires a shading reference image with the same size as the tile image.
Automatic	**Activated**: The function automatically calculates the reference image for shading correction from the input image.
Shading Reference	Only available in Batch mode and if **Global shading** is selected or **Camera shading** is selected and **Automatic** is deactivated. Selects a reference image for the shading correction.
Display Mode	
- Additive	In this mode the normalized reference image is subtracted from each camera frame. This influences the brightness of the image.
- Multiplicative	In this mode each camera frame is divided by the normalized reference image. This influences the contrast of the image. This is the default setting. The simulated/auto reference image is created by averaging up to 20 camera frames in the input image and running a lowpass filter on them.
Offset	Adjust the gray value that will be added on to the newly calculated gray values using the slider or input field. If this results in negative values, these are set to 0. Values that exceed the maximum gray value are set to the maximum gray value.

9.12.1.14 Slices Replacement

With this function you can replace one or more z-slices of a stack with the respective previous or following slice.

Parameter	Description
Replace with Next	Replaces the currently selected z-slice with the following one.
Replace with previous	Replaces the currently selected z-slice with the previous one.
Undo	Reverses the last replacement(s).
Redo	Restores the last undone change(s) for the replacement.

Parameter	Description
Replacement Table	Only visible if at least one replacement has been set. Displays information about the slice replacement and which **Z** is replaced.
– Z	Displays the index of the z-slice that gets replaced.
– Source	Displays the index of the z-slice which replaces **Z**.
– Replacement Type	Displays the type of replacement (**Next** or **Previous**).

See also

Replacing z-slices in a z-stack [358]

9.12.1.15 Sort SmartFIB Tiffs

This function allows you to select a folder with FIB-SEM tiffs and sort these files in separate subfolders according to channel name, number of pixels, image size, and spacing of the images, corresponding to slice thickness. The files are sorted into subfolders of the output folder according to their metadata. This means that each time one of the above mentioned metadata changes from one image to the subsequent image, a new sub folder is created. Thus subfolders with homogeneous metadata with respect to xyz-scaling and image size are created.
Note that this function only works if the tiff files have their default names assigned by the microscope (e.g. channel0_slice_0001.tiff or slice_0001.tiff)! All tiff files with other names are ignored by the function.

Parameter	Description
Input Folder	Selects the folder where the tiff files which should be sorted are saved. Click on [...] to open a file browser and navigate to the respective folder. Note that this function does not include images from subfolders, all images that you want to be sorted have to be in this input folder!
Output Folder	Selects the folder where the subfolders with the sorted tiff files are placed. Click on [...] to open a file browser and navigate to the respective folder. The function automatically creates subfolders in this folder.
File Operation	
– Copy	Copies the images into the output folder. Your data is duplicated and remains unchanged in the input folder.
– Move	Moves the images from your input folder into the output directory.
– Hard Link	Creates links in the output folder to each image file in the input folder, i.e. the space occupied by the images on the hard disk remains unchanged.

9.12.1.16 Z-Stack Alignment with ROI

This function allows you to automatically align the individual planes of a z-stack image if they are not positioned precisely above each other.

Parameter	Description
Quality	Selects the quality level that you want the function to work with. The calculation of the alignment is based on a so-called image pyramid. The higher the selected quality, the more levels of the image pyramid are used to calculate the alignment and the more precise the alignment will be. However, the higher the selected quality is, the slower the calculation of the alignment will get.
– Low	This is the most imprecise but also the fastest calculation of the alignment. It uses a low number of levels (2) of the image pyramid for the alignment calculation.
– Medium	This is a more precise but also a slower calculation of the alignment than the one before. It uses a medium number of levels (3) of the image pyramid for the alignment calculation.
– High	This is a more precise but also a slower calculation of the alignment than the two before. It uses a high number of levels (4) of the image pyramid for the alignment calculation.
– Highest	This is the most precise but also the slowest calculation of the alignment. It uses the highest number of levels of the image pyramid for the alignment calculation.
Registration Method	Selects the method which is used to align the images.
– Translation	The neighboring sections of the z-stack are shifted in relation to each other in the X and Y direction.
– Rotation	The neighboring sections of the z-stack are rotated in relation to each other.
– Translation + Rotation	The neighboring sections of the z-stack are translated and rotated in relation to each other.
Interpolation	Selects how the interpolation is performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.
Region	Selects which parts of the image should be considered for the calculation of the transformation matrix.
- Full	Considers the entire image for alignment.

Parameter	Description
- Rectangle Region	Allows you to draw a region of interest into the image. Only the image information of this region is then considered for the calculation of the transformation matrix for alignment. The resulting transformation matrix will be applied to the full image. After you have drawn a rectangle region into the image, you can see and change its coordinates with the **X/Y/W/H** input fields.
Crop output	**Activated:** The output image will have the same size as the input image. The image will be cropped in the case that pixels are shifted out of the area defined by the input image. **Deactivated:** The output image does not keep the size of the input image but incorporates also pixels that lie outside of the area defined by the original image.
Single Component	**Activated:** Displays the image channels. Selects one of the image channels which will be used to calculate the alignment transformation matrix, which then is also applied to the other channel(s). **Deactivated:** Calculates an alignment transformation matrix for each individual channel.

See also

Aligning z-planes automatically (based on a ROI) [358]

9.12.1.17 Z-Stack Equalization

This function equalizes the gray values throughout an entire image stack. It scales the intensities of all slices to a common mean intensity value and standard deviation. The goal is a harmonization of the gray value distribution within a z-stack. With this process, brighter image slices will get darker and darker slices will get brighter.

9.12.2 Deconvolution

9.12.2.1 Deconvolution (defaults)

This method allows you to use 4 different algorithms for deconvolution, without any further settings.
The following algorithms are provided in the **Parameter** tool:

Parameter	Description
Simple, very fast (Nearest Neighbor)	Executes the fast Nearest Neighbor method using default parameters.
Better, fast (Regularized Inverse Filter)	Executes the Regularized Inverse Filter algorithm for image enhancement.
Good, medium speed (Fast Iterative)	Executes the Fast Iterative restoration method.
Excellent, slow (Constrained Iterative)	Executes the Constrained Iterative quantitative restoration method.

9.12.2.2 Deconvolution (adjustable)

Deconvolution can be done either by using the computers CPU or by using a graphics card. Using a graphics card can speed up deconvolution processing quite dramatically. A NVIDIA graphics card is required which supports the CUDA processing library. Contact your sales representative for further details about supported graphic card models.

For a detailed overview of all deconvolution methods (and its combinations) see *Deconvolution Methods in ZEN [▸ 337]*. This method allows you to use and individually configure 4 different algorithms for deconvolution (short DCV). Under **Parameters** two tabs are available for detailed configuration:

- On the **Deconvolution** tab, you can select the desired algorithm and define the precise settings for it, see *Deconvolution tab [▸ 124]*.
- On the **PSF Settings** tab, you can see and change all key parameters for either generating a theoretically calculated PSF, or selecting an experimentally measured PSF, see *PSF Settings tab [▸ 130]*.

9.12.2.2.1 Deconvolution tab

Info

Expert knowledge is required for some of the settings. If you are in doubt, leave the settings unchanged.

Algorithm dropdown list

Here you can select the algorithm that is used. The following algorithms are available:

- Nearest Neighbor
- Regularized Inverse Filter
- Fast Iterative
- Constrained Iterative

Enable Channel Selection

Activated: Applies the settings on a channel-specific basis. This allows you to set parameters for each channel individually. You will see a separate, colored tab for each of the channels.

Deactivated: Applies the same settings to all channels of a multichannel image.

9.12.2.2.1.1 Normalization section

Here you can specify how the data of the resulting image are handled:

Parameter	Description
Clip	Sets negative values to 0 (black). If the values exceed the maximum possible gray value of 65636 when the calculation is performed, they are limited to 65636 (pixel is 100% white). Results from different input images can be quantitatively compared with each other.

Parameter	Description
Automatic	Normalizes the output image automatically. In this case the lowest value is 0 and the highest value is the maximum possible gray value in the image (gray value of 65636). The maximum available gray value range is always utilized fully in the resulting image. Results from different input images cannot directly be compared quantitatively with each other.

9.12.2.2.1.2 Set Strength Manually section

If you have selected the **Nearest Neighbor** algorithm, the checkbox is always activated.

Info

If you have selected the **Fast Iterative** algorithm, the checkbox is also always activated. Using the slider you can then enter the number of iterations used directly, as, in contrast to the other methods, no regularization is performed.

Activated: Enter the desired degree of restoration using the slider.

To achieve strong restoration and best contrast, move the slider towards **Strong**.

To achieve lower restoration but smoother results, move the slider towards **Weak**.

If the setting is too strong, image noise may be intensified and other artifacts, such as "ringing", may appear.

Deactivated: Determines the restoration strength for optimum image quality automatically. This is recommended for widefield and confocal images and is therefore deactivated by default.

The restoration strength is inversely proportional to the strength of so-called regularization. This is determined automatically with the help of Generalized Cross Validation (GCV).

9.12.2.2.1.3 Convergence History

Only visible if the **Show All** mode is activated.

This section is only visible if you have selected the **Fast Iterative** or **Constrained Iterative** algorithm.

The progress of the calculation is displayed here as line graph. Several quality parameters are measured for each iteration and once either an optimum or the maximum allowed number of iterations is reached, the processing is stopped. This display allows you to observe directly how the iterative method affects the available data. It also shows how many iterations have been used and how much time is being used per iterations.

9.12.2.2.1.4 Corrections section

To show the section in full, click on the **arrow** button .

Parameter	Description
Lamp Flicker	**Activated:** Analyzes the total brightness of each Z-plane. In the event of non-constant deviations in the total brightness between neighboring planes, a compensation factor is taken into account. Activate this function if you have acquired your images using an old fluorescent lamp that exhibits strong fluctuations/flickering in brightness.
Fluorescent Decay	**Activated:** Corrects bleaching of the sample during acquisition of the Z-stack. This function should only be activated for widefield images. Use it if your sample undergoes strong bleaching during acquisition.
Background	**Activated:** Analyzes the background component in the image and removes it before the DCV calculation. This can prevent background noise being intensified during DCV.
Bad Pixel Correction	**Activated:** Employs a fully automatic detection and removal of spurious or hot pixels (also known as stuck pixels) in an image stack which might interfere with the deconvolution result. It is based on the analysis of the gray level variance in the neighborhood of each pixel in the image. It is recommended to use this parameter only, if stuck pixels are observed in the input image.

9.12.2.2.1.5 Advanced Settings section

Only visible if the **Show All** mode is activated.

This section is only visible if you have selected the **Inverse Filter**, **Iterative (Fast)** or **Iterative** algorithm.

To show the section in full, click on the **arrow** button .

Depending on which algorithm you have selected, different advanced setting options are available. The relevant settings are described in the following chapters for each algorithm:

9.12.2.2.1.6 Advanced settings (Regularized Inverse Filter)

Regularization

Here you can select which frequencies in the image are taken into account during regularization:

Parameter	Description
Regularization	
- Zero Order	Regularization based on G-difference, modeled on Tikhonov, but accelerated.
- First Order	Regularization based on Good's roughness. Under certain circumstances, more details are extracted from noisy data. May be better suited to the processing of confocal data sets.

Parameter	Description
GPU Acceleration	Only available, when having installed a suitable (NVIDIA, CUDA based) graphics card. The checkbox is then activated by default. If unchecked, CPU processing is being used instead.

9.12.2.2.1.7 Advanced settings (Fast Iterative)

Parameter	Description
Likelihood	Here you can decide which likelihood calculation you want to work with:
- Poisson (Meinel)	The calculation according to Meinel works with one convolution per iteration and converges very fast, normally in 4-5 iterations. This method can also produce artifacts, however.
- Poisson (Richardson-Lucy)	The calculation according to Richardson-Lucy, on the other hand, normally requires hundreds of iterations and therefore takes considerably longer. This method is, however, somewhat more robust producing less artifacts. Precondition is however, that the PSF is known very well.
Regularization	For the **Poisson (Meinel)** calculation it is also possible to perform **zero order** (G-difference) regularization here as an option. This means, however, that the calculation will take considerably longer and the main advantage of the greater speed of Meinel is lost.
- None	No regularization is performed.
- Zero Order	Regularization based on G-difference, modeled on Tikhonov, but accelerated.
- Total Variation	Total Variation regularization denoises the input data but protects the edges of structures. This can improve results for noisy data but increases the computation time quite significantly.
Optimization	
- Numerical Gradient	If selected, an attempt is made to determine the trend of the iterations in advance and extrapolate this to the entire calculation. This can significantly speed up the calculation.
First Estimate	
- Input Image	The input image is used as the first estimate of the target structure (default).
- Last Output Image	The result of the last calculation is used to estimate the next calculation. This can speed up a calculation that is repeated using slightly different parameters.
- Mean of Input Image	No estimate is made, the mean gray level of the input image is being used. This is the most rigid application of deconvolution. It should be chosen for confocal images, where the data sampling can be quite sparse. The computation time will increase, but missing information can be recovered from the PSF.

Parameter	Description
Maximum Iterations	Here you can indicate the maximum permitted number of iterations that you want. In the case of Richardson-Lucy, you should allow significantly more iterations here.
Quality Threshold	Defines the quality level at which you want the calculation to be stopped. The percentage describes the difference in enhancement between the last and next-to-last iteration compared with the greatest difference since the start of the calculation. 1% is the default value. Lowering this can bring about small improvements in quality.
GPU Acceleration	Only available, when having installed a suitable (NVIDIA, CUDA based) graphics card. The checkbox is then activated by default. If unchecked, CPU processing is being used instead.
GPU Tiling	Only available for very large images which exceed the available graphic card memory. With this function the image is split up in smaller portions which fit into the memory of the graphic card. The function automatically determines into how many tiles the image must be split to allow maximum usage of the graphics card. The resulting tiles will automatically be stitched together for the final output result. If deactivated, tiling will not be done, however, in this case only certain sub-functions of deconvolution can run on the graphics card and the speed increase compared to CPU processing will be lower. The image quality might be higher than with tiling because there is no need for stitching.

9.12.2.2.1.8 Advanced settings (Constrained Iterative)

Parameter	Description
Likelihood	Here you can decide which likelihood calculation you want to work with:
- Poisson	Calculation according to Poisson, this is normally the correct noise model for microscopic images.
- Gauss	Calculation according to Gauss. If detector noise is dominant over sample noise, using a Gaussian noise model can be advantageous, however, this is rarely the case with modern microscopy systems.
Regularization	Here you can enter which frequencies in the image are taken into account during regularization:
- Zero Order	Regularization based on G-difference, modeled on Tikhonov, but accelerated.
- First Order	Regularization based on Good's roughness. Under certain circumstances, more details are extracted from noisy data. This regularization can sometimes produce better results for the processing of confocal data sets.
- Second Order	Regularization according to Tikhonov-Miller. Here higher frequencies are penalized more than in the case of Good's roughness. Results have a tendency to become overly smoothed.

Parameter	Description
Optimization	
- Analytical (Newton Raphson)	Here an attempt is made to optimize the iterations analytically. This option is usually faster but may also be somewhat less precise.
- Line Search	Searches rigorously and comprehensively for the minimum. It is therefore more robust, but the calculation takes longer. Line search is recommended for confocal data sets especially, if they are noisy as this can enforce convergence even for noisy and sparsely sampled data.
First Estimate	
- Input Image	The input image is used for the first estimate of the target structure (default).
- Last Output Image	The result of the last calculation is used to estimate the next calculation. This can speed up a calculation that is repeated using slightly different parameters.
- Mean of Input Image	No estimate is made for the next iteration. This is the most rigid application of deconvolution. It should be chosen for confocal images, where the data sampling can be quite sparse. The computation time increases increase, but missing information can be recovered from the PSF.
Maximum Iterations	Here you can indicate the maximum permitted number of iterations that you want.
Quality Threshold	Defines the quality level at which you want the calculation to be stopped. The percentage describes the difference in enhancement between the last and next-to-last iteration compared with the greatest difference since the start of the calculation. Lowering this can bring about small improvements in quality.
GPU Acceleration	Only available, when having installed a suitable (NVIDIA, CUDA based) graphics card. The checkbox is then activated by default. If unchecked, CPU processing is being used instead.
GPU Tiling	Only available for very large images which exceed the available graphic card memory. With this function the image is split up in smaller portions which fit into the memory of the graphic card. The function automatically determines into how many tiles the image must be split to allow maximum usage of the graphics card. The resulting tiles will automatically be stitched together for the final output result. If deactivated, tiling will not be done, however, in this case only certain sub-functions of deconvolution can run on the graphics card and the speed increase compared to CPU processing will be lower. The image quality might be higher than with tiling because there is no need for stitching.

9.12.2.2.2 PSF Settings tab

All key parameters for generating a theoretically calculated Point Spread Function ("PSF") are displayed here.

Info

Ordinarily, images that have been acquired using **ZEN** (of the *.czi type) automatically contain all microscope parameters, meaning that you do not have to configure any settings on this page. Most parameters are therefore grayed out in the display. It is possible, however, that as a result of an incorrect microscope configuration values may not be present or may be incorrect. You can change these here. The correction of spherical aberration can also be set here.

9.12.2.2.2.1 Microscope parameters section

The most important microscope parameters for PSF generation that are not channel-specific are displayed in this section.

Info

If you enter incorrect values, this can lead to incorrect calculations. If the values here are obviously wrong or values are missing, check the configuration of your microscope system.

Parameter	Description
Microscope dropdown list	Displays which type of microscope has been used. There are two main options: conventional microscope (also known as a widefield microscope) and confocal microscope, for which the additional pinhole diameter parameter applies.
NA Objective	Displays the numerical aperture of the objective.
Immersion	Displays the refractive index of the immersion medium. Please note that this can never be smaller than the numerical aperture of the objective. You can make a selection from typical immersion media in the dropdown list next to the input field.
Scale lateral	Displays the geometric scaling in the X/Y direction.
Scale axial	Displays the geometric scaling in the Z direction.
Override button	To change the input fields that are normally grayed out, click on the button. The input fields and dropdown lists are now active. The text on the button then changes to **Reset**. To restore the original values saved in the image, click on the **Reset** button.
Master Reset	Resets the metadata to the values which were originally stored in the image at time of acquisition. It reverts any changes made by pressing the **Override** button.

9.12.2.2.2.2 Advanced section

Only visible if the **Show All** mode is activated.

To show the section in full, click on the **arrow** button .

Phase Ring dropdown list

If you have acquired a fluorescence image using a phase contrast objective, the phase ring present in the objective is entered here. This setting has significant effects on the theoretical Point Spread Function ("PSF").

PSF generation dropdown list

There are two models for calculating the PSF:

Parameter	Description
Scalar Theory	The wave vectors of the light are interpreted as electrical field = intensity and simply added. This method is fast and is sufficient in most cases (default setting).
Vectorial Theory	The wave vectors are added geometrically. However, the calculation takes considerably longer.

Z-Stack

This field can only be changed if it was not possible to define this parameter during acquisition, e.g. because the microscope type was unknown. It describes the direction in which the Z-stack was acquired. Note that this setting is only relevant, if your are using the spherical aberration correction.

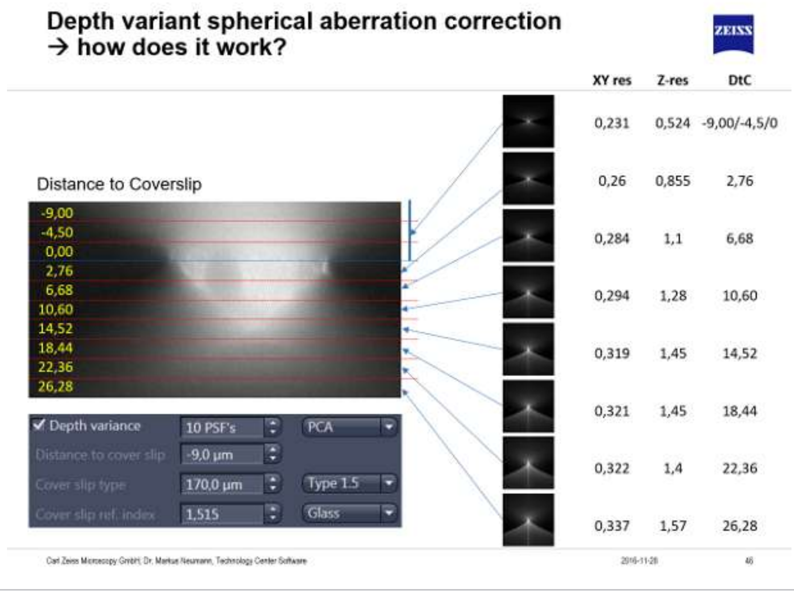
Parameter	Description
Descending	The Z-Stack descends away from the objective.
Ascending	The Z-Stack ascends towards the objective.

9.12.2.2.2.3 Aberration correction section

Only visible if the **Show All** mode is activated.

Here you can select whether you want spherical aberration to be taken into account and corrected during the calculation of the PSF. As with the other PSF parameters, most values are extracted automatically from the information about the microscope that is saved with the image during acquisition. The input option is therefore inactive.

Parameter	Description
Enable Correction	**Activated:** Uses the correction function. All options are active and can be edited.
Embedding medium	Here you can select the embedding medium used from the list.
Refractive index	Displays the refractive index of the selected embedding medium. Enter the appropriate refractive index if you are using a different embedding medium.

Parameter	Description
Manufacturer	Displays the manufacturer, if known.
Depth variance	When Aberration correction is activated, it is also possible to enable the creation of depth variant PSF's. This method allows for dramatic improvements in image restoration of thicker samples by creating axially variant theoretical PSF's as a function of the distance to the coverslip and the refractive index of the mounting medium. To use depth variant aberration correction activate the checkbox. In the spin box edit field you can define how many PSF's should be generated. The more PSF's you create, the better the results, but choosing many PSF's will increase the processing time. You should choose at least 3 PSF's. From the dropdown list you can choose between the PCA method (Primary Component Analysis, M. Arigovindan et al., 2005, IEEE Transactions on Image Processing 14. nr. 4 p.450ff) which is best suited for constrained iterative and fast iterative method and the Strata method (Myneni and Preza, Frontiers in Optics 2009, Optical Society of America, paper CThC4.), which is best for regularized inverse filter and Richardson Lucy iterative deconvolution.
Distance to cover slip	Displays the distance of the acquired structure from the side of the cover slip facing the embedding medium. Half the height of the Z-stack is assumed as the initial value for the distance from the cover slip. The value can be corrected if this distance is known. If possible, this distance should be measured. Note: Use **Ortho View** and the **Distance Measurement** option to define the distance of the sample to the coverslip. It is also important to estimate the position of the glass/embedding medium interface as precise as possible. If the z-stack extends into the coverslip, the determined range of the stack which reaches into the glass should be entered as a negative value. Example: Z-stack is 26 µm thick, glass/medium interface is positioned at 9 µm distance from the first plane of the stack. Resulting value for Distance to cover slip: - 9.0 µm. 

Parameter	Description
Cover slip type (Thickness and type)	Commercially available cover slips are divided into different groups depending on their thickness (0, 1, 1.5 and 2), which you can select from the dropdown list. Cover slips of the 1.5 type have an average thickness of 170 µm. In some cases, however, the actual values can vary greatly depending on the manufacturer. For best results the use of cover slips with a guaranteed thickness of 170 µm is recommended. Values that deviate from this can be entered directly in the **input field**.
Cover slip ref. index	Select the material that the cover slip is made of from the dropdown list. The corresponding refractive index is displayed in the input field next to it.
Working distance	Displays the working distance of the objective (i.e. the distance between the front lens and the side of the cover slip facing the objective). The working distance of the objective is determined automatically from the objective information, provided that the objective was selected correctly in the MTB 2011 Configuration program. You can, however, also enter the value manually.
Override	Only active if the **Enable correction** checkbox is activated. To reset the values, click on the **Reset** button.

9.12.2.2.2.4 Channel specific section

In this section you will find all settings that are channel-specific. This means that they may be configured differently for each channel.

Parameter	Description
Use external PSF checkbox	**Activated:** Uses an external measured PSF. You'll find an additional input window under **Image Parameters | Input** where you can choose the external PSF file. The software will check if the PSFs microscope parameters match with the input image. Deviations (10nm deviation in wavelength will be accepted) will make the software use a theoretical PSF.
Attach to input button	If an external PSF was selected you can attach the file to the input image. The saved input image will then contain the correct measured PSF. Usage of a theoretical PSF is possible as well for such an image. Just deactivate the **Use external PSF** checkbox.
Illumination display field	Displays the excitation wavelength for the channel dye [in nm] by using the peak value of the emission spectrum. The color field corresponds to the wavelength (as far as possible).
Detection display field	Displays the peak value of the emission wavelength for the channel dye. The color corresponds to the wavelength (as far as possible).

Parameter	Description
Sampling lateral display field	Depends on the geometric pixel scaling in the X/Y direction and displays the extent of the oversampling according to the Nyquist criterion. The value should be close to 2 or greater in order to achieve good results during DCV. As, in the case of widefield microscopes, this value is generally determined by the objective, the camera adapter used and the camera itself, it can only be influenced by the use of an Optovar. With confocal systems, the zoom can be set to match this criterion.
Sampling axial display field	Depends on the geometric pixel scaling in the Z direction and displays the extent of the oversampling according to the Nyquist criterion. The value should be at least 2 or greater in order to achieve good results during DCV. This value is determined by the increment of the focus drive during acquisition of Z-stacks and can therefore be changed easily.
Pinhole display field	Only available if a confocal microscope has been entered under the microscope parameters. Displays the size of the confocal pinhole in Airy units (AU).
NA Cond. display field	Only visible if the microscope is a **Conventional Microscope** and **Transmitted Light** has been selected as the illumination type. Displays the numerical aperture of the condenser with which transmitted-light acquisition was performed.

9.12.2.2.2.4.1 Microscope info section

Displays advanced microscope information that influences the form of the PSF in a channel-dependent way:

Parameter	Description
Illumination drop-down list	Here you can select the illumination method with which the data set has been acquired. In the event that a **Conventional Microscope** has been entered under the microscope parameters, the following options are available here: **Epifluorescence**, **Multiphoton Excitation** and **Transmitted Light**. In the case of confocal microscopes, **Epifluorescence** is the only option.
Image Formation	Displays whether the imaging was incoherent (**Conventional Microscope**) or coherent (**Laser Scanning Microscope**).
Lateral Resolution	Displays the lateral resolution of the calculated PSF.
Axial FWHM	Displays the FWHM (Full Width Half Maximum) as a measure of the axial resolution of the PSF.

9.12.2.2.2.4.2 PSF view section

This tool shows you the PSF that is calculated for a channel based on the current settings. If you select the **Auto Update** checkbox, all changes made to the PSF parameters are applied immediately to the PSF view. This makes it possible to check quickly whether the settings made meet your expectations.

1. To extract the PSF from the image, right click and select **PSF snapshot**.

The result is a new PSF document opened in the center screen area.

9.12.2.3 Creating a PSF - With Wizard and Without

The PSF Wizard combines two steps which are necessary for extracting experimental point spread functions (PSF) from Z-Stacks of subresolution fluorescent beads:

- A bead averaging step finds individual beads, presents them for inspection, allows you to select the ones you like and then creates an averaged combination of all selected beads. This stack shows a single bead which is, as a consequence of the averaging function, fairly free of noise.
- The averaged bead stack is then run through the **Create PSF** function which removes background and residual noise, correctly scales the PSF and also converts the stack into a 32-bit floating point format which is better suitable for the mathematical procedures used in deconvolution.

Prerequisite

✓ You have acquired a Z-Stack image. For more information, see *Measuring the PSF using subresolution beads [▸ 99]*.

✓ The use of the PSF wizard is activated.

1. Select the path to open the **Parameters** dialog box.
   → The functions **Use wizard** and **Bad Pixel Correction** are activated by default.
2. On the **Processing** tab, press the **Apply** button.
   → The PSF wizard opens and guides you throught the creation of the PSF.
   → If the **Use wizard** checkbox is not activated, the function shows the parameters for **Bead averaging**. Note that these parameters are only available in **Show all** mode. We recommend using the PSF wizard. The result of the wizard is a PSF file which you use in deconvolution for images acquired under the same conditions.

This method determines the position of fluorescent beads in a Z-stack image. If these beads are too close to one another they are excluded from the calculation. Beads which are far enough apart from one another are combined into a single bead, from which it is then possible to calculate a PSF using the **Create PSF** function (Processing / Utilities).

Description of the algorithm

This function consists of a series of steps before and after processing. The aim is to find beads that are far enough apart from one another. The processing steps are as follows:

- Select input image
- Image smoothing
- Segmentation
- Alignment of the center of the found beads
- Averaging of the beads

Parameters

- **Bead proximity**: Defines the distance between two neighboring beads in µm. A bead is excluded from the averaging if the distance to a neighboring bead is greater than the minimum distance set. A smaller value leads to the detection off fewer beads, albeit ones that are further apart, while a larger value leads to the detection of more beads, but with the risk that beads will partially overlap. Range between 2.1 and 20 µm.
- **Detection sensitivity**: determines the sensitivity with which beads are being detected. Range is between -5 to 5. Smaller Values lower the sensitivity excluding beads with weaker staining and lower signal to noise ratio, higher values include also beads with weaker staining.
- **Average Beads**: If activated, an image of an individual, averaged bead is produced. If deactivated, an image is produced in which each found bead is centered, but saved in the R dimension. A slider for Rotation (R dimension) appears on the **Dimensions** tab.

Bad Pixel Correction

This parameter employs a fully automatic detection and removal of spurious or hot pixels (also known as stuck pixels) in an image stack which might interfere with the PSF extraction procedure. It is based on the analysis of the gray level variance in the neighborhood of each pixel in the image. It is usually recommended to leave this parameter active.

9.12.3 Adjust

9.12.3.1 Background Subtraction

With this function you remove smooth background or correct uneven illumination. The implementation is adapted from the corresponding function in ImageJ and based on the “rolling ball” algorithm (S. Sternberg, “Biomedical Image Processing”, IEEE Computer, January 1983).

Parameters

Parameter	Description
Radius	Here you adjust the radius of the rolling ball in pixels. This value should be at least as large as the radius of the largest object in the image that is not part of the background. Larger values will also work unless the background of the image is too uneven.
Create Background only	If activated, an image which contains the detected background only is created. Use this image to subsequently perform manual corrections of the image background, e.g. using the image calculator function.
Do smoothing beforehand	If activated, a 3x3 pixel averaging is performed before analyzing the background. Use this option to ensure that the image data after subtraction will not be below the background.
Light Background	Use this option if your image contains bright background and dark objects.

9.12.3.2 Brightness/Contrast/Gamma

This method allows you to adjust the brightness, contrast and gamma value of an image.

Info

Unlike the adjustments that can be made on the **Display** tab, here the pixel values of the image are changed.

Parameters

Parameter	Description
Brightness	Adjust the desired brightness using the slider or input field. Changing the brightness means that each gray or color value is increased or decreased by the same value. The difference between the biggest and smallest gray or color value in the image remains the same, however.
Contrast	Adjust the desired contrast using the slider or input field. Changing the contrast means that the gray or color values are multiplied by a factor. The difference between the biggest and smallest gray or color value changes.
Gamma	Adjust the desired gamma value using the slider or input field. Changing the gamma value means that the gray or color values are multiplied by individual factors.

9.12.3.3 Color Balance

This method allows you to adjust the weighting of the individual color channels of a true color image.

Parameters

Parameter	Description
Range to Adjust	Here you can select the adjustment range for the color balance. There are 3 ranges available:
- Shadows	The settings relate to tones in the dark color range.
- Midtones	The settings relate to tones in the mid color range.
- Lights	The settings relate to tones in the light color range.
Cyan - Red	Adjust the desired color balance using the slider or input field.
Yellow - Blue	Adjust the desired color balance using the slider or input field.
Magenta - Green	Adjust the desired color balance using the slider or input field.

9.12.3.4 Color Temperature

This method allows you to adjust the color temperature of a true color image. Therefore use the **Temperature Delta** slider. A description of the slider can be found under *White Balance [▸ 141]*.

9.12.3.5 Histogram Equalization

This function enhances the contrast by linearizing the histogram of the image to equal area fractions in the histogram. The areas (pixel count multiplied by gray value range) of all gray values in the histogram of the result image are the same.

Parameter	Description
All Z	If activated, the function is applied to all Z planes.
All T	If activated, the function is applied to all time points.
High Threshold	The fraction of pixels that will be mapped to the highest gray value of the output image.
Lower Threshold	The fraction of pixels that will be mapped to gray value 0.

9.12.3.6 Hue/Saturation/Lightness

This method allows you to adjust the hue, saturation and brightness of a true color image.

Parameters

Parameter	Description
Hue	The value of the shift represents an angle on the color wheel. The values -180 and +180 therefore have an identical effect. Negative angles shift the color tone towards blue and positive ones shift it towards red. Adjust the desired shift in the color tone using the slider or input field.
Saturation	Saturation describes how intense the color of a pixel is. "Chromatic" is the maximum saturation, while "achromatic" describes colors that do not leave a color impression. Adjust the desired saturation using the slider or input field.
Lightness	Lightness describes how light or dark a pixel appears. The greatest difference is between black and white or between violet and yellow. Adjust the desired brightness using the slider or input field.

9.12.3.7 Shading Correction

This method allows you to improve images in which the quality has been impaired by uneven illumination or vignetting.

If you want to perform shading correction before an experiment (recommended) you have to use the shading correction function in the **Camera** tool in the *Post-Processing section [**673]*.

Parameter	Description
Shading Mode	
- Camera shading	Applies the Shading correction to each tile of a tile image.
- Global shading	Applies the Shading correction to the whole tile image. Requires a shading reference image with the same size as the tile image.

Parameter	Description
Automatic	**Activated**: The function automatically calculates the reference image for shading correction from the input image.
Shading Reference	Only available in Batch mode and if **Global shading** is selected or **Camera shading** is selected and **Automatic** is deactivated. Selects a reference image for the shading correction.
Display Mode	
- Additive	In this mode the normalized reference image is subtracted from each camera frame. This influences the brightness of the image.
- Multiplicative	In this mode each camera frame is divided by the normalized reference image. This influences the contrast of the image. This is the default setting. The simulated/auto reference image is created by averaging up to 20 camera frames in the input image and running a lowpass filter on them.
Offset	Adjust the gray value that will be added on to the newly calculated gray values using the slider or input field. If this results in negative values, these are set to 0. Values that exceed the maximum gray value are set to the maximum gray value.

9.12.3.8 Shading Reference from Tile Image

With this method you can create shading reference images for multi-channel tile images.
Note: Z-stack and time series images cannot be processed with this function! The best results are achieved with tiles larger than 200 images.

Parameters	Description
All Scenes	Only available for multi-scene images. Selects all scenes for processing.
Selected Scenes	Only available for multi-scene images and if **All Scenes** is deactivated. Selects the scene for processing.
Merge Channels	Creates a single multi-channel-reference-image (with the same channel order) in one step.
Adjust per Channel	Only available for images with multiple channels. **Activated:** You can adjust the settings for every channel separately. To use a channel select **Process Channel** or select **Skip Channel** if you do not want a channel to be processed. The settings **Save directly as Shading Reference** and **Channel-specific** are applied separately for each channel. If you want to use the same settings for all channels deactivate the checkbox.

Parameters	Description
Save directly as Shading Reference	**Activated**: The software creates the shading reference image and stores it directly to the Calibration Manager (Shading Reference). The software provides no possibility to check the image before they will be saved, thus it is recommended to deactivate the checkbox and execute this function. The system will create the reference images and present it to you. If the images have a good quality, you can activate the checkbox and run the function again. Note that, if you activate this option it is important that you check afterwards the system messages (the "i" in the lower part of the screen). If e.g. the resulting shading reference image is too dim the system will not use it as valid shading reference image, this will be shown under the system messages. Also if the import of the reference database were successful this will be shown!
Channel-specific	**Activated**: The software performs channel-specific shading correction. In this case the fluorescence filter block used is saved with the shading file. The following components will be considered: Contrasting method and condenser, fluorescence filter, magnification: Objective and Optovar; camera bit depth and RGB/BW mode, camera type and port position. **Deactivated**: The system creates an **All Channel** calibration and perform an objective specific shading correction. The following components will be considered: Magnification (Objective and Optovar); Camera bit depth and RGB/BW mode, and camera type and port position.
Multiply Factor	Here you can apply a multiply factor, thus the software will multiply the pixel intensity for each pixel of the shading reference image by this value. If you use an own sample it is mostly the case that the images are very dim and the intensity does not reach the value needed to be used within the shading reference calibration manager, thus it will be rejected.
Auto Adjust Intensity	**Activated:** Automatically calculates the multiply factor based on the gray values of the image and the needed gray values for using it in the shading reference calibration manager. If activated, the setting for **Multiply Factor** has no influence anymore on the image generation.
Apply Gaussian Filter	**Activated:** A Gaussian filter is applied after the averaging of the field of views from a tiled image is done. This enables to smooth the shading reference image. The **Sigma** factor defines the strength of the smoothing. Use this filter and the Sigma factor very carefully as it could remove also features which are real shading structures. This feature could be recommend if the number of tiles in the scanned image is low and cannot be increased for certain reasons.

9.12.3.9 Stack Correction

This method allows you to improve the quality of Z-Stack images that have been affected by bleaching effects during acquisition.

Parameters

Under **Correction** you can select the desired correction mode or a combination of the modes.

Parameter	Description
Decay	This mode compensates the bleaching effect.
Flicker	This mode compensates the flicker of the lamp voltage.
Background	This mode reduces background noise.

9.12.3.10 White Balance

This method allows you to adjust the white balance of an image.

Parameters

Parameter	Description
Automatic	**Activated**: The white spot is calculated automatically from the image data.
White Spot	Only visible if the **Automatic** checkbox is deactivated. Define the white spot by clicking on the **Pick..** button. The mouse pointer then changes to a pipette symbol. Use it to click on a white region of your input image. The coordinates and color values of the selected white spot are displayed next to the button.
Temperature Delta	Adjust the delta that will be added on to the newly calculated color values. Negative values reduce the color temperature, while positive values increase it. A value of 1 corresponds to 10 Kelvin.

9.12.4 Edges

9.12.4.1 Gradient Max

This method performs a gradient filtering. Based on the sum of a 2x2 matrix in the X-and Y-direction, a gradient image is calculated and using the larger of the two components. The edges are darker than that of the method **Gradient Sum**.

9.12.4.2 Gradient Sum

This method performs a gradient filtering. Based on the sum of a 2x2 matrix in the X-and Y-direction, a gradient image is calculated. The edges are brighter than that of the method **Gradient Max**.

9.12.4.3 Highpass

This method performs high-pass filtering. The high pass filter is defined as the difference between the original image and the low-pass filtered original.

Parameter	Description
Normalization	Depending on the image processing function you have selected not all choices are available in the list.
- Clip	Gray levels that exceed or fall below the specified gray value range are automatically set to the lowest/highest gray value (black or white). The effect corresponds to underexposure or overexposure. This means that in some cases information is lost.
- Automatic	Automatic normalization of gray values to the available gray value range.
- Wrap	If the result is greater than the maximum gray value of the image, the value maximum gray value +1 is subtracted from it.
- Shift	Normalizes the output to the value gray value + max. gray value/2.
- Absolute	Values are used positive only.
Count	Here you set the number of repetitions. I.e. the number of times the function is applied sequentially to the respective result of the filtering. The effect is increased correspondingly.
Kernel Size	You can set the filter size in the x-, y-and z-direction, symmetrically around the subject pixel. This should be the size of the transition region between objects and background match.

9.12.4.4 Laplace

This function performs a Laplace highpass filter on an image.

The calculation is based on a 3x3x3 Laplace operator in all directions. The function does not show smooth gray value changes very well.

9.12.4.5 Local variance

This method is an edge filter, which calculates the variance of each pixel with its neighboring pixels by the lateral filter size.

Parameter

Kernel Size in X/Y

Here you set the matrix size in X / Y symmetrically around the pixel. This determines the degree of smoothing effect in the X / Y direction.

9.12.4.6 Roberts

This method calculates a gradient image using the Roberts filter matrix. Large gray value differences between neighbors are shown as light gray values. No changes are indicated by a value of 0 (black). Edges are thinner than with the **Sobel** method.

9.12.4.7 Sobel

Sobel calculates a gradient image using a Sobel filter.

This method indicates gray value changes in the image. Large differences between neighbors are displayed as bright gray values, no changes are indicated by a value of 0 (zero). The pixels in the output image are calculated with the Sobel differential operator on the basis of a 3x3x3 fold of the input image.

9.12.5 Geometric

9.12.5.1 Change Orientation

With this function you can easily change the image orientation.

Parameters

Parameter	Description
Orientation	
- Flip Horizontally	Flips the image horizontally.
- Flip Vertically	Flips the image vertically .
- Rotate 90 CW	Rotates the image by 45 degrees clockwise (CW).
- Rotate 90 CCW	Rotates the image by 45 degrees counter clockwise (CCW).
- Rotate 180	Rotates the image by 180 degrees.
- Mirror at +45 Diagonal	Mirrors the image at +45 degrees diagonal.
- MIrror at -45 Diagonal	Mirrors the image at -45 degrees diagonal.

9.12.5.2 Channel Alignment

Using this method it is possible to automatically align the individual channels of a multi-channel image correctly to one another.

Parameters

Parameter	Description
Registration Method	Here you can select the method (or a combination of these) to be used to align the images.
- Translation	The neighboring sections of the Z-stack image are shifted in relation to each other in the X and Y direction.
- Rotation	The neighboring sections of the Z-stack image are rotated in relation to each other.
- Iso Scaling	The magnification is adjusted from section to section.

Parameter	Description
- Skew Scaling	The neighboring sections of the Z-stack image are corrected for skewness / shearing.
- Affine	The neighboring sections of the Z-stack image are shifted in the X and Y direction, rotated and the magnification is adjusted from section to section.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Quality	Here you can select the quality level that you want the function to work with.
- Low	Highest speed with low image quality.
- Medium	High speed with medium image quality.
- High	Low speed with high image quality.
- Highest	Lowest speed with highest image quality.
Interpolation	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.

9.12.5.3 Channel Alignment (Extended)

Using this method it is possible to automatically align the individual channels of a multi-channel image correctly to one another.

Parameters

Parameter	Description
Load transformation	If activated, the result of a previous transformation can be loaded. Click on ... to select an according ***.xm**l file.
Save transformation	If activated, the result of the transformation process is saved in an ***.xml** file for later use.

Parameter	Description
Registration Method	Here you can select the method (or a combination of these) to be used to align the images.
- Translation	The neighboring sections of the Z-stack image are shifted in relation to each other in the X and Y direction.

Parameter	Description
- Rotation	The neighboring sections of the Z-stack image are rotated in relation to each other.
- Iso Scaling	The magnification is adjusted from section to section.
- Skew Scaling	The neighboring sections of the Z-stack image are corrected for skewness / shearing.
- Affine	The neighboring sections of the Z-stack image are shifted in the X and Y direction, rotated and the magnification is adjusted from section to section.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Quality	Here you can select the quality level that you want the function to work with.
- Low	Highest speed with low image quality.
- Medium	High speed with medium image quality.
- High	Low speed with high image quality.
- Highest	Lowest speed with highest image quality.
Interpolation	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.

9.12.5.4 Color-coded Projection

The Color-coded projection function generates a maximum intensity projection image along the z-, or time-dimension of a multidimensional data set. Instead of using the colors assigned to the channels, it displays the position in z or in time with a color gradient.

Parameters

Parameter	Description
Palette	Sets the color-pattern for the projection.
ROI	Specifies the range of the dimension (chosen below) which will be used for the projection.
Dimension	Selects whether to perform the projection along the time (**T**) or along the z-axis (**Z**). Only dimensions which are available in your data set are shown.

Parameter	Description
Quantitative	**Activated:** Uses a quantitative method which first creates a maximum intensity projection and then determines at which plane a pixel was located. After that it attributes a color of the LUT to the pixel. This method can sometimes produce abrupt transitions, but the pixel color representing depth or time corresponds correctly to the colors in the annotation. **Deactivated:** This method attributes a color of the LUT to each plane and then separates the result into RGB channels. Subsequently it creates a maximum intensity projection of each RGB channel and then combines the results into one image. Note that while this might result in a more pleasantly looking image, the method can produce colors which do not correspond to colors in the annotation!

9.12.5.5 Image Overlay

With this function you can align (or overlay) two images that are displaced in relation to each other. It is also possible to make the individual planes of a z-stack image congruent, in the event that they are not lying exactly on top of one another.

You can define 3 related points (**Input Pixel**) in both the input image and in the reference image (**Reference Pixel**) that is displaced in relation to it. Therefore click interactively on conspicuous points, which are present in both images. If you click **Apply** the function calculates the Output image, in which the new fitting points have the same coordinates as in the Input image.

Parameters

Parameter	Description
Input Pixel 1 - 3	If you click on the corresponding buttons you can define the 3 input pixel points. The selected point is shown in the graphics plane. This serves as an aid to orientation when you are clicking on the reference points.
Reference Pixel 1 - 3	If you click on the corresponding buttons you can define the 3 reference pixel points.
Interpolation	Here you can specify how the rotation influences the neighboring pixels.
- Linear	The rotated pixel is given the gray value calculated from the linear combination of the gray values of the pixel closest to it and this pixel's nearest neighbor.
- Cubic	The rotated pixel is given the gray value resulting from a polynomial function of the pixel that is closest to it.

9.12.5.6 Mirror

This method allows you to flip an image horizontally or vertically. In the case of multidimensional images, such as Z-stack or time lapse images, you can also use the mirror method to reverse the sequence of the relevant dimension.

Parameters

Parameter	Description
Display Mode	
- Horizontal	Flips the image horizontal
- Vertical	Flips the image vertical
- T/ Z	Only visible if input image is a multichannel image. Reverses sequence of the sections (Z) or time points (T)

9.12.5.7 Orthogonal Projection

With this method you can extract specific parts of the image of three-dimensional images. This is accomplished with three alternative projection planes, frontal in the XY direction, sagittal in YZ direction or transverse in XZ direction as seen from the observer of the image. You can choose between different projection methods, all have in common is that the pixels are analyzed by the observer along an imaginary projection beam. You can also determine the thickness of the projection planes, and thus the projection depth.

Parameters

Parameter	Description
Projection Plane	Here you choose the type of the projection plane (Frontal X/Y, Transverse (X/Z), Sagittal (Y/Z).
Method	
- Maximum	Uses the brightest pixel along the projection beam.
- Minimum	Uses the darkest pixel along the projection beam.
- Average	Calculates the average of all pixel along the projection beam.
- Weighted average	This method is related to the calculation of the extended depth of focus. It prefers structures with more lateral sharpness along the projection beam. The output image contains more significant details.
- Standard deviation	Calculates the standard deviation of pixel grey values along the projection beam.
Start position	Here you adjust the starting position of the project plane (in pixel units or z-stack positions depending on the chosen projection plane). The maximum range results automatically of the size of the input image.
Thickness	Here you adjust the thickness of the cutting plane (in pixel or z-stacks depending on the chosen projection plane). The maximum range results automatically of the size of the input image.

9.12.5.8 Resample

This method allows you to change the size of an image in every dimension. You can either enlarge or reduce the image size.

Parameters

Parameter	Description
Third Dimension	Only visible, if there is a third dimension in the input image and/or **Show all** mode is activated. Here you can select how you want the function to work in the case of multidimensional images.
- 2D Slices	The function is used in a 2-dimensional fashion only for multidimensional images such as time lapse or z-stacks.
- Z, T or C	Here you can select to which additional dimension the functions should be applied to.
Adapt sizes	**Activated:** The size of the output image is adjusted in accordance with the settings for the scaling. **Deactivated:** The output image has the same size as the input image. Depending on the image size and rotation angle, partial areas of the input image may not be visible in the output image.
Adjust per Channel	Only visible if your input image is a multi-channel image. **Activated:** You can adjust the parameters for each channel individually.
Interpolation	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.

Controls

Parameter	Description
Scaling in X	Adjust the desired scaling for X using the slider or input field.
Scaling in Y	Adjust the desired scaling for Y using the slider or input field.
Scaling in Z	Adjust the desired scaling for Z using the slider or input field.
The following parameters are only visible if the **Adapt sizes** checkbox is deactivated:	
Shift in X	Enter the shift in the X direction using the slider or input field.
Shift in Y	Enter the shift in the Y direction using the slider or input field.
Shift in Z	Enter the shift in the Z direction using the slider or input field.

9.12.5.9 Rotate

With this method you can rotate images by defined angles. This function was especially developed for rotating complex (multi-dimensional) images in the available image dimensions. Therefore the function can be a little bit slower but offers more settings for the rotation. For simple, 2-dimensional rotations we recommend to use the **Rotate 2D** function which is usually lots of faster.

Parameter

Parameter	Description
Third Dimension	Only visible, if there is a third dimension in the input image and/or **Show all** mode is activated. Here you can select how you want the function to work in the case of multidimensional images.
- 2D Slices	The function is used in a 2-dimensional fashion only for multidimensional images such as time lapse or z-stacks.
- Z, T or C	Here you can select to which additional dimension the functions should be applied to.
Adapt sizes	**Activated:** The size of the output image is adjusted in accordance with the settings for the scaling. **Deactivated:** The output image has the same size as the input image. Depending on the image size and rotation angle, partial areas of the input image may not be visible in the output image.
Adjust per Channel	Only visible if your input image is a multi-channel image. **Activated:** You can adjust the parameters for each channel individually.
Interpolation	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.

Parameter	Description
Angle	Enter the angle by which you want the input image to be rotated around using the slider or input field. Positive angles rotate the images clockwise.
Angle X	Enter the angle by which you want the input image to be rotated on the X axis using the slider or input field.
Angle Y	Enter the angle by which you want the input image to be rotated on the Y axis using the slider or input field.

Parameter	Description
Angle Z	Enter the angle by which you want the input image to be rotated on the Z axis using the slider or input field.
The following parameters are only visible if the **Adapt sizes** checkbox is deactivated:	
Center X	Enter the X coordinate of the center of the rotation using the slider or spin box/input field. The value 0 means that the image is rotated around its center point. Negative values mean that the center of the rotation in the image is shifted to the left in relation to the image's center point. Positive values shift the center to the right.
Center Y	Enter the Y coordinate of the center of the rotation using the slider or spin box/input field. The value 0 means that the image is rotated around its center point. Negative values mean that the center of the rotation in the image is shifted downwards in relation to the image's center point. Positive values shift the center upwards.
Center Z	Enter the Z coordinate of the center of the rotation using the slider or spin box/input field. The value 0 means that the image is rotated around its center point. Negative values mean that the center of the rotation in the image is shifted forwards in relation to the image's center point. Positive values shift the center backwards.

9.12.5.10 Rotate 2D

With this method you can easily rotate an image clockwise around its center axis. Simply set the desired angle with the slider. Of course you can enter the angle value in the input field directly. To perform the rotation click on the **Apply** button on top of the **Processing** tab.

Parameter	Description
Angle	Enter the angle by which you want the input image to be rotated around using the slider or input field. Positive angles rotate the images clockwise.
Interpolation	
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.
Edge Smoothing	**Activated**: Performs edge smoothing.
PaveWholePlane	**Activated**: Paves the whole resulting plane with tiles; there might be empty tiles. **Inactivated**: Creates only tiles which contain also parts of the image.

Parameter	Description
ProcessMask	**Activated**: Rotates also the mask of valid/invalid pixels. **Inactivated**: Ignores the mask of valid/unvalid pixels. Pixels previously marked as invalid will have an intensity of zero after rotation but are not marked as invalid.
IncludeGraphic Elements	**Activated**: Rotates also the graphical elements together with the image. **Inactivated**: Rotates only the image, but not the graphical elements.

9.12.5.11 Sample Down

With this function you can reduce the size of an image in a flexible way. The reduction is performed along with an averaging of the respective dimension. If the parameters are set to 1, the corresponding dimension is not modified.

Parameters

Parameter	Description
Average Pixels X	Here you adjust how many pixels are averaged in the lateral X dimension to calculate the output image. The size of the output will be smaller by this factor.
Average Pixels Y	Here you adjust how many pixels are averaged in the lateral Y dimension to calculate the output image. The size of the output will be smaller by this factor.
Third Dimension	If your input image has a third dimension you can select it here for resampling. ▪ **Z** (sections of a Z-Stack) ▪ **T** (time points of a time series) ▪ **C** (Channels) or ▪ **H** (Phases). ▪ If **2D Slices** is chosen, the third dimension will not be re-sampled.

9.12.5.12 Shift

This method allows you to shift the content of an image in the direction of the 3 axes X, Y and Z. To adjust the shift use the respective sliders or input fields under **Parameters.**

Parameter	Description
Third Dimension	Only available if **Show All** is activated an if the acquired image is three dimensional. Selects the third dimensional shift.
– 2D Slices	The third dimension is shifted by 0 which means the image is only shifted in X / Y direction.
– Z	The shift is in Z direction.

Parameter	Description
– C	Shifts the channel. If you have a 32 channel image, the first 30 channels are copied/ interpolated into the last ones (for an 32 channel lambda image this would result in a shift to the red spectrum). When selected, the checkbox **Adjust per channel** is not available.
Adjust per Channel	Only available for images with multiple channels. Opens a list with the channels to allow an individual adjustment of each channel.
– Process Channel	Shifts this channel according to the input.
– Skip Channel	Skips this channel when processing. This channel will not be in the output image.
– Copy Channel	This channel is copied into the output image without a shift.
Shift in X	Selects the shift of the content in X direction.
Shift in Y	Selects the shift of the content in Y direction.
Shift in Z	Selects the shift of the content in Z direction.

9.12.5.13 Stage Alignment

The images are registered by using the stage calibration points from the meta data.

Parameter	Description
Interpolation	Here you can select how you want interpolation to be performed.
- Nearest Neighbor	The output pixel is given the value of the input pixel that is closest to it.
- Linear	The output pixel is given the value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the value resulting from a polynomial function of the input pixels closest to it.

9.12.5.14 Stitching

This method allows you to align the individual tiles of a tile image with one another automatically and correctly.

Parameters

Parameter	Description
Inplace	The stitching is applied to the original image.
New Output	A new image is generated as a result of the stitching process. The original image is not modified.
Fuse Tiles	Only visible when the button **New Output** is selected. **Activated**: All individual tile images are fused together after alignment. **Deactivated**: The individual tile images are aligned but not fused.
Correct shading	Only visible when the button **New Output** is selected. **Activated:** Applies a shading correction (Multiplicative mode, see *Shading Correction [▸ 138]*) to each image of prior to stitching. Select from the dropdown list which reference should be used for shading correction:
- Automatic	The function automatically calculates a reference image from the input image.
- Reference	The function uses an existing reference image. This must be selected in addition to the input image in the input tool of the image parameters section. In Batch mode, a reference image must be defined beneath.
Select Reference Image	Only visible in Batch mode and if **Reference** is selected as shading correction. Selects a reference image for the shading correction.

Select dimension references for stitching

Only visible for multidimensional input images.

Select here a reference dimension (one channel, one z-position, one time point) from your multidimensional data set. This reference dimension is either stitched exclusively (no other planes of the dimensions are stitched) or serves as reference when stitching all planes of the dimensions.

Parameter	Description
Get all dimensions from 2d view	Reads the current planes of the dimensions from the 2D view.
Z-Position	Selects a z-position for the 2D image.
Time	Selects a time point for the 2D image.
Channels	Selects a channel for the 2D image.
Illumination	Only visible for dual side illuminated images. Selects an illumination side for the 2D image.

Parameter	Description
View	Only visible for Multiview images. Selects a view for the 2D image.

Stitch multiple dimensions section

Only visible for multidimensional input images. Under the respective dimension you can select which planes of the dimensions should be considered for stitching.

Parameter	Description
All by reference	Only the reference plane (2D image) for this dimension is taken into consideration for calculating the stitch. All other planes are stitched accordingly and appear in the output image.
Reference only	Only the selected reference plane (2D image) of this dimension is stitched. No other planes appear in the output image.
All individually	All planes of this dimension are stitched and appear in the output image. The stitch is calculated individually for each plane.

Parameters

Parameter	Description
Edge Detector	
- Yes	An edge detector is applied to localize image edges. This may improve the stitching result.
- No	No edge detector is applied.
Minimal Overlap	Sets the extent of the area of minimal overlap (in %) of the individual tiles
Max Shift	Sets the maximal extent of the shift (in %) of the individual tiles to one another.
Comparer	Here you can select how the conformance of the tiles in the overlapping regions is evaluated.
- Basic	Basic comparison (faster).
- Best	Elaborate comparison (slower).
- Optimized	Optimized comparison.
Global Optimizer	Select here which tile overlaps are evaluated.
- Basic	Only one overlap per tile is evaluated.
- Best	All overlaps of a tile are evaluated.

9.12.5.15 Z-Stack Alignment

This method allows you to bring the individual planes of a Z-stack image into line if these are not positioned precisely one above the other. This is the case, for example, when you acquire Z-stacks at an oblique angle using a stereo microscope.

Parameters

Parameter	Description
Registration Method	Here you can select the method (or a combination of these) to be used to align the images.
- Translation	The neighboring sections of the Z-stack image are shifted in relation to each other in the X and Y direction.
- Rotation	The neighboring sections of the Z-stack image are rotated in relation to each other.
- Iso Scaling	The magnification is adjusted from section to section.
- Skew Scaling	The neighboring sections of the Z-stack image are corrected for skewness / shearing.
- Affine	The neighboring sections of the Z-stack image are shifted in the X and Y direction, rotated and the magnification is adjusted from section to section.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Quality	Selects the quality level that you want the function to work with. The calculation of the alignment is based on a so-called image pyramid. The higher the selected quality, the more levels of the image pyramid are used to calculate the alignment and the more precise the alignment will be. However, the higher the selected quality is, the slower the calculation of the alignment will get.
– Low	This is the most imprecise but also the fastest calculation of the alignment. It uses a low number of levels (2) of the image pyramid for the alignment calculation.
– Medium	This is a more precise but also a slower calculation of the alignment than the one before. It uses a medium number of levels (3) of the image pyramid for the alignment calculation.
– High	This is a more precise but also a slower calculation of the alignment than the two before. It uses a high number of levels (4) of the image pyramid for the alignment calculation.
– Highest	This is the most precise but also the slowest calculation of the alignment. It uses the highest number of levels of the image pyramid for the alignment calculation.
Interpolation	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.

9.12.6 Morphology

Morphology functions apply structure elements to images. A structure element is like a stencil with holes. When the stencil is placed on an image then only some pixels are visible through the holes. The gray values of these pixels are collected and their external gray value (minimum or maximum) is computed.

This external gray value is assigned to that pixel of the resulting image which corresponds to the place of the origin of the stencil on the input image. When the stencil is placed at all positions of the input image, all pixels of the resulting image are thus assigned. When bigger structure elements are required than those which are provided, these can be achieved by iterating the small elements using the **Count** parameter.

The following functions are available:

Function	Description
Erode	Shrinks bright structures on a darker background in the input image. Thin connections between structures and small structures itself will disappear.
Dilate	Expands bright structures on a darker background in the input image. Small gaps between structures are filled and those structures become connected.
Open	First erodes (Erode class) the bright structures on a darker background in the input image, then it dilates (Dilate class) the result by the same number of steps. Thus it separates bright structures on a darker background, but approximately keeps the size of the structures.
Close	First dilates (Dilate class) the bright structures on a darker background in the input image, then it erodes (Erode class) the result by the same number of steps. Thus it connects bright structures on a darker background, but approximately keeps the size of the structures.
Top Hat (White)	Computes the difference between the original image and the image produced by an open operation (Open class). Bright structures which were flattened by the opening are strengthened in the result. This is like putting a top hat with the size of the open operation upon the structure and keep only the part inside the hat.
Top Hat (Black)	Computes the difference between the original image and the image produced by a close operation (Close class). Dark structures which were flattened by the closing are strengthened in the result. This is like lifting a top hat with the size of the close operation beneath the structure from the dark side and keep only the part inside the hat.
Gradient	Computes the difference between the dilated image and the eroded image (Dilate and Erode class). Since a point in the dilated image has the maximum gray value and the corresponding point in the eroded image has the minimum gray value within the structure element the difference is zero for regions of constant gray values and gets bigger for steeper gray value ramps or edges.

Function	Description
Watersheds	Computes the barriers between catchment basins of local minima in the gray valued input image. A local minimum is a connected plateau of points from which it is impossible to reach a point of lower gray value without first climbing up to higher gray values. A catchment basin of a local minimum is a connected component which contains that minimum and all downstream points to it. A downstream is a path of points along which gray values are monotonically descending. Thus all catchment basins of local minima are expanded until they collide with another catchment basins. At those point barriers (watersheds) are built up. The output image is binary and contains all watersheds. If the 'Basins' flag is set, the catchment basins themselves are in the output image as uniquely labeled connected components without any border lines.
Grey Reconstruction	Works mainly as an iterated dilation (Dilate class) of the image, but with a constraint image as a second input image. After every dilation step the pixel wise minimum of the dilated image and the constraint image is computed and gives the next image to be dilated. The computation stops automatically when all the just dilated pixels are bigger than the corresponding ones in the constraint image.

Parameters

Parameter	Description
Structure Element	Here you select the desired structure element. Following elements are available: Horizontal, Diagonal 45°, Vertical, Diagonal 135°, Cross, Square, Octagon
Count	Here you can adjust the number of repetitions to define the size of the structure element.
Binary	Only available for **Erode**, **Dilate**, **Open** and **Close** function. **Activated:** Creates a binary image. The calculation will be faster.

9.12.7 Sharpen

9.12.7.1 Delineate

This method enhances the edges of individual regions in an image. It corrects the halo effect and only affects edges.

Parameters

Parameter	Description
Threshold	Enter the threshold value for edge detection using the slider or spin box/input field. The threshold value should correspond roughly to the gray value difference between objects and the background.
Size	Enter the size of the edge detection filter using the slider or spin box/input field. The value should correspond to the size of the transition area between objects and the background.

9.12.7.2 Enhance Contour

This method allows you to enhance contours in an image and emphasize regions in which gray values change. The function is suitable for visually emphasizing fine structures in an image.

Parameters

Parameter	Description
Strength	Here you can adjust the factor for increasing edge enhancement.
Normalization	Here you can select how the gray/color values that exceed or fall short of the value range should be dealt with.
- Clip	Automatically sets the gray levels that exceed or fall short of the pre-defined gray value range to the lowest or highest gray value (black or white). The effect corresponds to underexposure or overexposure. In certain circumstances some information may therefore be lost.
- Automatic	Normalizes the gray values automatically to the available gray value range.
- Wrap	If the result is larger than the maximum gray value of the image, the maximum gray value + 1 is deducted from this value.
- Shift	Normalizes the output to the value "gray value + maximum gray value/2".
- Absolute	Converts negative gray levels into positive values.

9.12.7.3 Extended Depth of Focus

Using this method you can combine the sharp regions from the individual sections of a Z-stack image to form a single image. This enables you to display a considerably larger depth of field than is possible on a microscope.

Parameters

Parameter	Description
Method	
- Wavelets	A wavelet transform is used to detect the sharpest areas in the images.
- Contrast	For the this method the value is the difference between the brightest and the darkest pixel value within the “Kernel”.
- Maximum Projection	Images with the brightest and darkest pixels are generated first. Of these images the image with the higher variance is used as the resulting image.
- Variance	For this method the variance of the pixel values is calculated within the “Kernel”.

Parameter	Description
Z-Stack Alignment	Here you can select whether you want the Z-stack image to be aligned before the calculation and with what quality level. If you want to acquire images with a stereo microscope, the images are displaced against each other. This displacement can be corrected. The higher the quality of alignment is selected, the longer is the calculation. Select **No Alignment**, if you want to acquire images with a compound microscope.
- No Alignment	The Z-stack image is not aligned before the calculation. You should select this setting if the Z-stack image has not been acquired using a stereo microscope.
- Normal	High speed with normal image quality.
- High	Low speed with high image quality.
- Highest	Lowest speed with best image quality.

9.12.7.4 Unsharp Mask (Sigma)

Using this method you can increase the impression of sharpness in an image and consequently obtain an image display that is richer in detail. The function allows contrasts at small structures and edges to be enhanced in a targeted way.

Parameter	Description
Strength	Enter the strength of the Unsharp Masking using the slider or spin box/input field. The higher the value selected, the greater the extent to which small structures are enhanced.
Sigma	Adjusts the sigma value derived from *Gauss [▸ 161]*. Reduces noise in an image. Each pixel is replaced by a weighted average of its neighbors. The neighboring pixels are weighted in accordance with a two-dimensional Gauss bell curve.
Color Mode	Select the desired color mode from the dropdown list.
- RGB	Calculates the sharpness for each color channel individually. The color saturation and the color of structures may be changed and color noise may occur.
- Luminance	Only calculates the sharpness on the basis of the brightness signal detected. This mode does not show any color noise and changes the color saturation accordingly.
Threshold Mode	Here you can select a setting from the dropdown list for calculating the boundary between the sharpened image regions. It is only effective if the value for the **Lower Threshold Value** parameter is not equal to 0 or the value for the **Upper Threshold Value** parameter is not equal to 100.
- None	No adjustment takes place.
- Binary	The transition follows the threshold values.
- Linear	Calculates a linear course.

Parameter	Description
Threshold Low	Enter the lower threshold value using the slider or spin box/input field. This determines the lower limit from which existing contrast structures are changed.
Threshold High	Enter the upper threshold value using the slider or spin box/input field. This prevents the existing strong contrasts in the image from being increased further unnecessarily.
Clip To Valid Bits	**Activated:** The value range of the gray/color values of the output image is adjusted to the value range of the input image.

See also

Using Direct Processing [▸ 346]

9.12.8 Smooth

9.12.8.1 Binomial Filter

This method allows you to reduce noise in an image. Each pixel is replaced by a weighted average of its neighbors. The weighting factors are calculated from the binomial coefficients in accordance with the filter size. The binomial filter is very similar to a Gaussian filter in its effect.

Parameter

Parameter	Description
Kernel Size	Her you can adjust the size of the filter matrix. If the **Show All** mode is activated you can adjust the values in X ,Y and Z direction individually.

9.12.8.2 Denoise

This method removes noise from images using a real or a complex wavelet transformations. The process of denoising an image can be broken down into the following three parts:

- Calculate the wavelet transform of the noisy image.
  The wavelet transformation can be calculated by the method **Real Wavelets** and **Complex Wavelets**.
- Modify the noisy wavelet coefficients.
  This is done by using bivariate shrinkage with local variance estimation (thresholding). [Bivariate Shrinkage with Local Variance Estimator, Levent Sendur and Ivan W. Selesnick, IEEE Signal Processing Letters, Vol. 9, No. 12, December 2002]
- Compute the inverse transform using the thresholded coefficients.

Parameter	Description
Method	
- Complex wavelets	The Dual Tree Complex Wavelet transform provides better results due to the fact that it is nearly direction invariant and makes more directional sub bands available. The results will be less prone to block-artefacts. However, this method is computationally more intense and therefore takes longer.

Parameter	Description
- Real wavelets	The real wavelet transform only considers three sides (XYZ) and is therefore faster. However, the result can show block artefacts.
Strength	Here you adjust the strength with which the function is applied.

See also

Using Direct Processing [346]

9.12.8.3 Gauss

This method allows you to reduce noise in an image. Each pixel is replaced by a weighted average of its neighbors. The neighboring pixels are weighted in accordance with a two-dimensional Gauss bell curve.

Parameter	Description
Sigma	Here you can adjust the sigma value. If the **Show All** mode is activated you can adjust the values in each dimension individually.

9.12.8.4 Lowpass

This method allows you to reduce noise in an image. Each pixel is replaced by the average of its neighbors. The size of the area of the neighboring pixels considered is defined by a quadratic filter matrix. The modified pixel is the central pixel of the filter matrix.

Parameter

Parameter	Description
Count	Enter the number of repetitions using the slider or input field. The function can be applied several times in succession to the result of the filtering. This intensifies the effect accordingly.

Parameter	Description
Kernel Size	Her you can adjust the size of the filter matrix. If the **Show All** mode is activated you can adjust the values in X ,Y and Z direction individually.

9.12.8.5 Median

This method allows you to reduce noise in an image. Each pixel is replaced by the median of its neighbors. The size of the area of the neighboring pixels considered is defined by a quadratic filter matrix. The modified pixel is the central pixel of the filter matrix. The median is the middle value of the gray values of the pixel and its neighbors sorted in ascending order.

Parameter	Description
Kernel Size	Here you can adjust the size of the filter matrix. If the **Show All** mode is activated you can adjust the values in X ,Y and Z direction individually.

9.12.8.6 Rank

This method performs a rank order filtering. The gray levels of the resulting image is determined by calculating the ranking within the matrix of the **filter size** in the **X** and **Y** directions. Even numbers are automatically set to the next odd number. A low value for the **rank value** enlarges dark areas, a higher value will increase bright areas of the image.

9.12.8.7 Sigma

This method allows you to reduce noise in an image. Each pixel is replaced by the average of its neighbors. The size of the area of the neighboring pixels considered is defined by a quadratic filter matrix. The modified pixel is the central pixel of the filter matrix. To calculate the average, only the gray values that lie within a defined range (+/- sigma) around the gray value of the central pixel are taken into consideration. As a result, fine object structures are not blurred; only the gray levels in image regions that belong together are adjusted.

Parameter

Parameter	Description
Sigma	Enter the sigma value using the slider or input field.

Parameter	Description
Kernel Size	Her you can adjust the size of the filter matrix. If the **Show All** mode is activated you can adjust the values in X ,Y and Z direction individually.

9.12.8.8 Single Pixel Filter

With this function you can remove single pixel phenomena, such as those that occur in the case of clocking induced charge with EMCCDs and as radio telegraph signal noise with CMOS sensors. It is a filter which analyzes the input image and removes pixels, whose intensity value diverges strongly from the median intensity of its neighboring pixels.

The filter analyzes the input image and removes pixels that are "much" larger than the median of their neighbors. The algorithm works as follows:

1. Sort all 9 pixel in a 3x3 neighborhood.
2. Determine the median intensity value of the sorted pixels.
3. Multiply the median by the threshold factor to get the limit.
4. If the center pixel intensity is larger than the limit, replace the pixel with the median.

Larger values of the threshold factor increase the value of the intensity limit and decrease the number of pixels that are replaced. The default value of 1.5 is arbitrary, but seems to remove charge induced noise from images acquired using cameras with EM gain capabilities. This filter can also be used to remove hot pixels from images.

Parameter

Parameter	Description
Threshold	Here you adjust the threshold value.

9.12.9 Time Series

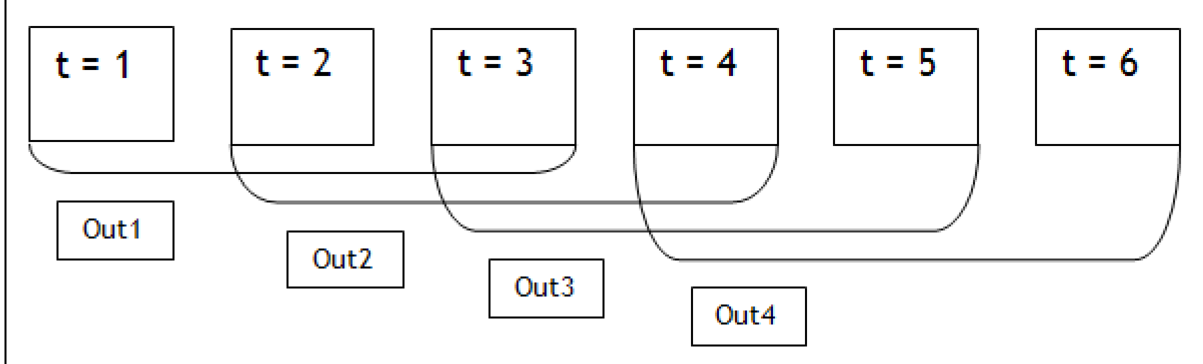
9.12.9.1 Gliding Average

With this function a smoothing effect can be achieved due to averaging out noise. It therefore calculates the gliding average of a time series image, taking into account the defined number of time points according to the following schematic:

Input Image: SizeT = 6

Averaging Length: AvL = 3

Output image: SizeT(output) = SizeT – AvL + 1 = 6 -3 +1 = 4



Parameter

Parameter	Description
Average Length	Specifies the number of images used to determine the mean value. The maximum value correlates with the number of time points.
Scaling Factor	The preset value is 1. Values > 1 can be applied for images with low intensity. In this case all pixel values are multiplied by the specified factor.

9.12.9.2 Kymograph

This method creates a Kymograph. The input image has to be a time series image containing a graphical element (e.g. a line, arrow, curve or polygon) which is not locked.

Parameter

Parameter	Description
Graphic tool	Here you see all graphic tools which can be used. Choose the desired graphic tool from the list. Note that the tool must be selected in the image as well.
Width	Here you adjust the graphic tools width (in pixel). This determines which pixels are used to calculate the average gray value along the width.

9.12.9.3 Time Alignment

Using this function you can automatically align individual time points in order to compensate for shifts between time points.

Info

For the alignment function to work the presence of immobile and clearly distinguishable object structures in the time series is required. Also, when aligning z-stacks over time, you should always use the z dimension from the Third Dimension drop down list. Otherwise each z-plane would be aligned over time potentially leading to z-stack artefacts.

Parameter

Parameter	Description
Registration Method	Here you can select the method (or a combination of these) to be used to align the images.
- Translation	The neighboring sections of the Z-stack image are shifted in relation to each other in the X and Y direction.
- Rotation	The neighboring sections of the Z-stack image are rotated in relation to each other.
- Iso Scaling	The magnification is adjusted from section to section.
- Skew Scaling	The neighboring sections of the Z-stack image are corrected for skewness / shearing.
- Affine	The neighboring sections of the Z-stack image are shifted in the X and Y direction, rotated and the magnification is adjusted from section to section.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Quality	Here you can select the quality level that you want the function to work with.
- Low	Highest speed with low image quality.
- Medium	High speed with medium image quality.
- High	Low speed with high image quality.
- Highest	Lowest speed with highest image quality.
Interpolation	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.

9.12.9.4 Time Concatenation

This function joins two images to form a new time series image. Select the desired images in the **Input** tool and click **Apply**.

Info

Images with varying dimensions will be put into different blocks by this function. The resulting image document shows a **Block** slider in the **Dimensions** view block.

9.12.9.5 Time Differential

This function calculates the first and second order differential of a time lapse image according to the following formula and schematic:

First Order Differential:

Output[t] = Input[t+1] – Input[t-1]

-> the difference between consecutive pixels is not calculated so that the output is not directional. The first order differential represents the **Speed**.

Second Order Differential:

Output[t] = Input[t-1] + Input[t+1] – 2 x Input[t]

->Second order differential is also known as the "Laplacian" and represents the **Acceleration**. It enhances the fine details in the image (including noise). The smoothing kernel helps reduce this noise.

Input:	t_1	t_2	t_3	t_4
First Order Output:		$t_2 = t_3 - t_1$	$t_3 = t_4 - t_2$	
Second Order Output:		$t_2 = t_3+t_1-2t_2$	$t_3 = t_4+t_2-2t_3$	

Parameter

Parameter	Description
Derivative	Here you can select whether to calculate the first (speed) or second (acceleration) order differential.
Smoothing	Indicates the iterative, binominal smoothing filter. This reduces noise in the differential images, whilst retaining maximums and minimums. Value range: 0 – 50
Normalization	Defines what to do with negative values resulting from the calculation. ▪ **Clip:** negative values are set to 0. ▪ **Absolute:** negative values are used positively.

9.12.9.6 Time Stitching

This function stitches heterogeneous **CZI** images together to create a new, single homogeneous time series containing all dimensions and time points in their proper order. This differs from the **Time Concatenate** function, which simply pastes one time lapse series to the end of another without regard for the proper time order or channel content.

Missing images can either be filled with copies of the previous valid image in the series or filled with black images.

When combining Z-stack time series with non-Z-stack time series a choice can be made between either using only the center plane of the Z-stack or creating an extended focus projection of the Z-stack before stitching the images together.

Parameter

Parameter	Description
Fill Missing with	
- Previous	Fills a missing dimension index with a copy of the last existing image from that index.
- Black	Fills a missing dimension index with a black image.
Z-Stacks	
- Collapse (EDF)	Reduces a z-stack with an extended focus function to a single plane image which is then added to the output.
- Collapse (Center Plane)	Only uses the center plane from a z-stack for the output image.
- Expand	Copies the z-stack to the output unchanged, fills the missing indices according to the setting in the **Fill Missing with** dropdown list.

9.12.10 Arithmetics

9.12.10.1 Add

This function adds the two images **Input1** and **Input2** pixel by pixel and generates the **Output** image. Note that a resulting gray value may be greater than the maximum gray value of the image.

Parameter

Parameter	Description
Normalization	Depending on the IP function you have selected not all choices are available in the list.
- Automatic	Automatic normalization of gray values to the available gray value range.
- Clip	Automatically sets the gray levels that exceed or fall below the specified gray value range to the lowest/highest gray value (black or white). The effect corresponds to underexposure or overexposure. This means that in some cases information is lost.

Parameter	Description
- Shift	Normalizes the output to the value gray value + max. gray value/2.
- Wrap	If the result is greater than the maximum gray value of the image, the value maximum gray value +1 is subtracted from it.

9.12.10.2 Add Constant

This function adds the factor **Addend** to each pixel of the **Input** image and generates the **Output** image.

To work useful with this function, the pixel values of the input image must be in float format. If images of type 8 bit, 16 bit, 24 bit and 48 bit are used as input image, the output images are of the same type, i.e. the output value will be clipped to an integer value. Negative values are set to 0, values higher than the maximum gray value are set to the maximum possible gray value of the image.

Parameter

Parameter	Description
Addend	Here you adjust the addend.

9.12.10.3 Average

The function calculates the average of the two images **Input1** and **Input2** pixel by pixel and generates the **Output** image.

9.12.10.4 Combine

This function calculates the linear combination of two images on a pixel basis.

Both **Input** images are first multiplied by the specified factor and then added together. The brightness of the **Output** image can then be adjusted. The combination of two images can be used to reduce noise, for example. This is achieved by acquiring several images of the same scene and subsequently combining them.

Parameters

Parameter	Description
Factor 1	Weighting factor for input image 1. **Value range**: -1,00 ... +1,00
Factor 2	Weighting factor for input image 2. **Value range**: -1,00 ... +1,00
Offset	Offset between input images 1 and 2.

9.12.10.5 Divide

This function divides the images **Input1** by **Input2** pixel by pixel and generates the **Output** image.

Parameter

Parameter	Description
Factor	Here you adjust the scaling factor by which the result of the division is multiplied. Using this factor it is possible to keep the gray values of the output image within the range of 0 to the maximum gray value. Values that are greater than the maximum gray value are in any case limited to the maximum gray value. Negative values are set to 0. **Value range**: -20.000 ... +20.000

9.12.10.6 Exponential

This function calculates the exponential function of the **Input** image pixel by pixel and generates the **Output** image.

To work useful with this function, the pixel values of the input image must be in float format. If images of type 8 bit, 16 bit, 24 bit and 48 bit are used as input image, the output images are of the same type, i.e. the output value will be clipped to an integer value. Negative values are set to 0, values higher than the maximum gray value are set to the maximum possible gray value of the image.

9.12.10.7 Invert

This function additively inverts the gray values of the input image into the output image. Bright pixels will become darker and vice versa. To adjust the output range the parameter **Operand** is used. The actual mathematical operation is then: output-gray value = constant - input-gray value. Negative results are clipped to 0 and overflow results are clipped to the maximum possible gray value.

9.12.10.8 Logarithm

This function calculates the logarithm of the **Input** image pixel by pixel and generates the **Output** image.

To work useful with this function, the pixel values of the input image must be in float format. If images of type 8 bit, 16 bit, 24 bit and 48 bit are used as input image, the output images are of the same type, i.e. the output value will be clipped to an integer value. Negative values are set to 0, values higher than the maximum gray value are set to the maximum possible gray value of the image.

9.12.10.9 Maximum

The function calculates the minimum values of the two images **Input1** and **Input2** pixel by pixel.

9.12.10.10 Minimum

The function calculates the maximum value of the two images **Input1** and **Input2** pixel by pixel.

9.12.10.11 Multiply

This function multiplies the two images **Input1** and **Input2** pixel by pixel and generates the **Output** image.

Parameter

Parameter	Description
Factor	Here you adjust the scaling factor by which the result of the multiplication is divided. Using this factor it is possible to keep the gray values of the **Output** image within the range of 0 to the maximum gray value. Values that are greater than the maximum gray value are in any case limited to the maximum gray value. Negative values are set to 0. **Value range**: -20,000 ... +20,000

9.12.10.12 Multiply Constant

This function multiplies each pixel of the **Input** image with a adjustable **Factor** and generates the **Output** image.

To work useful with this function, the pixel values of the input image must be in float format. If images of type 8 bit, 16 bit, 24 bit and 48 bit are used as input image, the output images are of the same type, i.e. the output value will be clipped to an integer value. Negative values are set to 0, values higher than the maximum gray value are set to the maximum possible gray value of the image.

Parameter

Parameter	Description
Factor	Here you adjust the factor to be multiplied.

9.12.10.13 Reciprocal

This function computes the reciprocals of the gray values in the input image into the output image. Bright pixels will become darker and vice versa. To adjust the output range the parameter "factor" is used. The actual mathematical operation is then: output-gray value = factor / input-gray value. Negative results are clipped to 0 and overflow results are clipped to the maximum possible gray value.

9.12.10.14 Square

This function calculates the square of the **Input** image pixel by pixel and generates the **Output** image.

To work useful with this function, the pixel values of the input image must be in float format. If images of type 8 bit, 16 bit, 24 bit and 48 bit are used as input image, the output images are of the same type, i.e. the output value will be clipped to an integer value. Negative values are set to 0, values higher than the maximum gray value are set to the maximum possible gray value of the image.

9.12.10.15 Square Root

This function calculates the square root of the **Input** image pixel by pixel and generates the **Output** image.

To work useful with this function, the pixel values of the input image must be in float format. If images of type 8 bit, 16 bit, 24 bit and 48 bit are used as input image, the output images are of the same type, i.e. the output value will be clipped to an integer value. Negative values are set to 0, values higher than the maximum gray value are set to the maximum possible gray value of the image.

9.12.10.16 Subtract

This function subtracts the two images **Input1** and **Input2** pixel by pixel and generates the **Output** image. Note that a resulting gray value may be less than 0.

Parameter

Parameter	Description
Normalization	Depending on the IP function you have selected not all choices are available in the list.
- Automatic	Automatic normalization of gray values to the available gray value range.
- Clip	Automatically sets the gray levels that exceed or fall below the specified gray value range to the lowest/highest gray value (black or white). The effect corresponds to underexposure or overexposure. This means that in some cases information is lost.
- Shift	Normalizes the output to the value gray value + max. gray value/2.
- Wrap	If the result is greater than the maximum gray value of the image, the value maximum gray value +1 is subtracted from it.

9.12.11 Segmentation

9.12.11.1 Canny

Canny detects edges in an image. This function detects relatively thick contours at the edge of bright regions.

Parameter

Parameter	Description
Sigma	Degree of smoothing of input image before detection of edges. A Gauss filter is used as a smoothing function. The smoothing factor can be used to influence the sensitivity of recognition. If a low value is set, lots of edges are detected. Fewer edges are detected with a high value. If the value 0 is set, no smoothing is performed.

Parameter	Description
Threshold	Steepness of the edges to be detected. Low values mean "flat" edges with a wide transition area between two regions. In this case lots of edges are detected. If high values are used, fewer edges are detected, as only steep transition areas are interpreted as edges.

9.12.11.2 Marr

This mehtod detects edges or regions in an image. In contrast to **Valleys** and **Canny**, here a Laplace filter is calculated, followed by smoothing using a **Gauss** filter, and the edges (Display Mode | Edges) or regions (Display Mode | Regions) are detected.

Parameter

Parameter	Description
Sigma	Degree of smoothing of input image before detection of edges or regions. A Gauss filter is used as a smoothing function. The smoothing factor can be used to influence the sensitivity of recognition. If a low value is set, lots of edges are detected. Fewer edges are detected with a high value. If the value 0 is set, no smoothing is performed.
Display Mode	
- Edges	Region edges are detected.
- Regions	The regions, not just their edges, are detected.

9.12.11.3 Threshold

This function performs a segmentation based on the definition of a brightness range (separated according to color channels (red, green, blue)) for the regions to be segmented. All pixels whose color values lie within the defined color range are marked as region pixels in the resulting image. All the pixels whose color values lie outside the defined color range are marked as background pixels (black).

In the resulting image, the color values of the region pixels can either be set permanently to white or adopted unchanged. If you set the region pixels permanently to white, the result is a binary image, which can then be used as a mask image for a subsequent automatic measurement.

Parameters

Parameter	Description
Level Low	Determines the lower brightness threshold for the regions to be segmented. All the pixels whose gray values lie below this threshold value are marked as background pixels (black).
Level High	Determines the upper brightness threshold for the regions to be segmented. All the pixels whose gray values lie above this threshold value are marked as background pixels (black).

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Create binary	**Activated:** The resulting image is a binary image. Pixels within the calculated gray level range are set to the maximum gray value (white), whilst pixels outside it are set to the gray value 0. **Deactivated:** The resulting image is of the same type as the input image. Pixels within the calculated gray level range are set to the original gray value. Pixels outside it are set to 0.
Invert result	**Activated:** Inverts the effect of the function. The segmented regions will be given the value 0, and all other pixels the gray value white or the gray value/color of the input image.

9.12.11.4 Threshold (auto)

This method performs an automatic gray value segmentation. The function calculates the two minimums in the individual channels in the gray value histogram of the input image (**Input**) and uses these for the segmentation.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Method	
- Otsu	For all possible threshold values, the Otsu method calculates the variance of intienisities on each side of the respective threshold. It minimizes the sum of the variances for the background and the foreground.
- Maximum Peak	Separates background and foreground pixels at the maximum value of the histogram.
- Iso Data	The threshold value lies in the middle between two maximums in the histogram.
- Triangle Threshold	The threshold value is calculated from the sum of the average and three times the sigma value of the histogram distribution.
- Three Sigma Threshold	

Parameters

Parameter	Description
Create binary	**Activated:** The resulting image is a binary image. Pixels within the calculated gray level range are set to the maximum gray value (white), whilst pixels outside it are set to the gray value 0. **Deactivated:** The resulting image is of the same type as the input image. Pixels within the calculated gray level range are set to the original gray value. Pixels outside it are set to 0.
Invert result	**Activated:** Inverts the effect of the function. The segmented regions will be given the value 0, and all other pixels the gray value white or the gray value/color of the input image.

9.12.11.5 Threshold (dynamic)

This method performs an adaptive gray value segmentation. This procedure is particularly well suited to the segmentation of small structures against a varying background.

The function initially applies a low pass filter and then subtracts this low-pass-filtered image from the input image. The effect of this function mainly depends on the size of the filter matrix: Select a low value for **Size** to segment small regions or regions with low gray value contrast from the background. Select a higher value for **Size** to segment larger regions from the background.

Parameters

Parameter	Description
Kernel Size	Matrix size of the low pass filter in x- and y-direction symmetrically around the pixel in question. Determines the extent of the smoothing effect. As the affected pixel is at the center, the edge length of the filter matrix is always an odd number. If an even number is entered via the keyboard, the value is always set to the next highest odd number.
Threshold	This value defines the gray value difference between the regions to be detected and the background. Segmented pixels are set to the maximum gray value (white), whilst other pixels are set to the gray value 0.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Create binary	**Activated:** The resulting image is a binary image. Pixels within the calculated gray level range are set to the maximum gray value (white), whilst pixels outside it are set to the gray value 0. **Deactivated:** The resulting image is of the same type as the input image. Pixels within the calculated gray level range are set to the original gray value. Pixels outside it are set to 0.
Invert result	**Activated:** Inverts the effect of the function. The segmented regions will be given the value 0, and all other pixels the gray value white or the gray value/color of the input image.

9.12.11.6 Valleys

This method detects dark lines (gray value valleys) on a bright background and contours between bright regions.

Parameter

Parameter	Description
Sigma	Degree of smoothing of input image before detection of valleys. The smoothing factor can be used to influence the sensitivity of recognition. If a low value is set, lots of valleys are detected. Fewer valleys are detected with a high value.
Threshold	Curvature at the valley bottom high/low. Depending on the setting made, weakly pronounced valleys are also detected, or only ones that are strongly pronounced.

9.12.12 Binary

9.12.12.1 And

This method performs a bit-by-bit AND calculation for the input images (**Input1** and **Input2**). This function is particularly useful for the masking of images. All the pixels that are white in input image 1 AND input image 2 are set to white in the resulting image. Pixels that are white in only one of the two input images become black.

9.12.12.2 Apply Mask

This tool enables you to isolate features in an image and to suppress image areas not of interest using a mask image.

Parameter	Description
Input	The input image from which you wish to isolate features or suppress areas not of interest
Mask	The mask image that is applied to the input image The mask is laid on top of the input image. Image regions of the input image **in1** where the mask is white remain unchanged, image regions where the mask is black are blacked out and suppressed. Both images are aligned at the upper left corner. If the mask image is smaller than the input image **in1**, the mask is applied only to part of the input image, beginning at the upper left corner. The rest of the input image remains unchanged.

9.12.12.3 Distance

This method creates a distance-transformed image (distance map, distance image) from a binary image. The Euclidean distance to the next background pixel (gray value 0) is calculated for each pixel within the white regions of the binary image (input image), and coded as a gray value. Bright pixels (high gray values) indicate a long distance to the background.

9.12.12.4 Exoskeleton

This method generates an image with the skeleton of the influence zone of regions. The background in the **Input** image is analyzed, and the skeleton of the influence zones of the objects is determined. This is then saved as a binary image in the **Output** image.

9.12.12.5 Fill Holes

This method fills holes in regions. Holes are structures that have the gray scale value 0, and is completely surrounded by pixels with a gray value equal to 0. Of regions outside of the image, it is assumed that they are black. Black areas that touch the edge of the image are preserved, therefore, even if they are surrounded by a contour.

9.12.12.6 Label Image

Assigns a gray value to each object in a binary image.

Parameters

Parameter	Description
Label Background	**Activated:** Assigns gray values to the background objects with con-nectivity 4. **Deactivated:** Assigns gray values to the background objects with Connectivity 8.

9.12.12.7 Mark Regions

This function marks binary regions of the input image. For each region in the input image, a check is performed to establish whether a pixel has been set in the marker image.

Parameters

Parameter	Description
Select Marked	**Activated:** Copies the marked region into the output image. **Deactivated:** Copies the unmarked region into the output image.

9.12.12.8 Not

This function performs a binary "not" operation on all bits of the binary representation of an input pixel's gray value. A 0-bit in the input pixel results in an 1-bit in the corresponding output pixel an 1-bit in the input gets a 0-bit in the output. For integral image types the resulting output gray value is the difference of the maximum possible gray value minus the input gray value, but for float image type the results are strange due to the inhomogeneous float format.

9.12.12.9 Or

This method performs a bit-by-bit OR calculation for the **Input1** and **Input2** images. This function can be used to combine binary masks or regions. All the pixels that are white in input image 1 OR input image 2 are set to white in the resulting image. This means that all the white pixels in the two input images are white in the resulting image.

9.12.12.10 Scrap

This method removes regions within a certain area.

Parameters

Parameter	Description
Minimum Area	Here you adjust the minimum area in Px.
Maximum Area	Here you adjust the maximum area in Px.
Select in Range	

9.12.12.11 Separation

Using this function you can attempt to separate objects that are touching (and that you have been unable to separate using segmentation) automatically.

Parameters

Parameter	Description
Separation Mode	
- Morphology	This method separates objects by first reducing and then enlarging them, making sure that once objects have been separated they do not merge together again.
- Watersheds	With this method you can separate objects that are roughly the same shape. This method may however result in the splitting of elongated objects.
Count	Enter how often the method is applied successively to the result at the location of the separation, using the slider or input field.

9.12.12.12 Thinning

This method thins objects to a line of single pixel thickness.

Parameters

Parameter	Description
Thinning Element	Select the desired thinning method here.
- Arcelli	Applies thinning in accordance with the Arcelli method.
- Levialdi	Applies thinning in accordance with the Levialdi method.
Count	Sets the number of repetitions. This means that the function is applied a number of times in succession to the filtering result. This increases the effect accordingly. The value range is from 1...256.
Prune	Cuts off the ends of the thinned lines.
Converge	If activated, the function is automatically repeated until all regions will be deleted by the next erosion step.
AxioVision Compatibility	Performs the function exactly like in AxioVision to achieve identical results.

9.12.12.13 Ultimate Erode

This function works in the same way as normal erosion. Structures in the input image are reduced. Thin connections between regions are separated. The difference between this function and normal erosion is that structures are eroded until they would be deleted by the next erosion step. With erosion, the pixel in question is set to the gray value 0 (black) in the resulting image. For regions (pixels) at the image edge, the assumption is that the pixels outside the image are white.

Parameters

Parameter	Description
Structure Element	Here you select the preferred direction of morphological change (e.g. Cross, Diagonal).
Count	Here you set the number of repetitions. This means that the function is applied a number of times in succession to the filtering result. This increases the effect accordingly.
Converge	If activated, the function is automatically repeated until all regions will be deleted by the next erosion step.

9.12.12.14 Xor

This method performs a bit-by-bit Xor calculation for the **Input1** and **Input2** images. This function can be used to combine binary masks or regions. All the pixels that are white in input image 1 or input image 2 are set to white in the resulting image. Pixels that are white in both input images are set to black.

9.12.13 Utilities

9.12.13.1 Add Channels

This method allows you to combine two input images that have different channels but otherwise have the same dimension (Z-stack, time series, tile, scene). An image is produced that contains all the channels of the input images.

If the two input images differ from one another in the dimensions Z-stack, time series, tiles or scene, input image 1 and input image 2 are copied into the output image as two separate blocks.

9.12.13.2 Airyscan Processing

With this function you can access the superresolution data in images acquired with Airyscan.

Info

Please note that starting with ZEN 2.5 blue edition, the black border of the processed image is automatically removed. Hence the resulting image will be smaller by 24 pixels in X and Y dimension.

Parameters

Parameter	Description
3D Processing	This option is only available for images with 5 or more z-positions. If activated, this option improves the resolution in axial and lateral direction. The data set needs to have at least 5 z-sections acquired with an optimal step size. 3D Processing is slower than 2D Processing. For 3D Processing, the whole z-stack (single channel and time point) needs to fit into the physical memory.

Parameter	Description
2D SR Processing	This function is available for 2D images only. It enhances the 2D resolution. Note this only results in increased superresolution when images are acquired with optimal settings and sufficient signal.
Auto Filter	If activated, a suitable **Super Resolution** parameter for the Airyscan processing is automatically determined for the selected data set. To manually adjust the **Super Resolution** parameter, deactivate the checkbox. Then determine suitable values by using the corresponding function in the Airyscan viewer in the **Airyscan** view. Note that the preview is only suitable for 2D Airyscan processing. A preview for 3D Airyscan processing is not available. For adjusting 3D processing parameters, you should first process your data set once using the Auto Filter and then check the value that was actually applied by the Airyscan processing function. This value is stored in the metadata of the processed image and can be accessed using the **Info** view. **Note:** High strength might look attractive at some images, Z planes or color channels, but other filtering artefacts might occur which appear like small rings in the image. Also the results will become very sharp, but grainy. So carefully check your image data in order to avoid such artefacts.
Adjust per Channel	Only visible, when the **Auto Filter** is deactivated. Only available for images with two or more Airyscan channels. If activated, you can manually set channel-specific Airyscan processing parameters.
Strength	Use this option for an increased (high) or decreased (low) strength of the automatically assigned filter value. This is especially useful for 3D processing, as the 2D preview of the processing filter value in the Airyscan viewer does not allow to conclude the result after a 3D data processing. The increment of this parameter is ± 0.4 compared to the standard auto Airyscan processing. This setting is not available when manual processing strength is selected.

9.12.13.3 ApoTome deconvolution

This method accepts **ApoTome** raw data only.

It was derived from the **Deconvolution** modul and is available in every licensed version of the software. It contains settings and parameters which make sense for an Apotome deconvolution only.

Parameter

Find the description of the parameters under: Deconvolution (adjustable) parameters . This method is available for batch processing as well.

9.12.13.4 ApoTome RAW Convert

This method accepts ApoTome raw data only. The settings are similar to the ones on the **ApoTome** tab (view option for ApoTome images). The function is also available for batch processing, which makes it easy to convert a series of ApoTome RAW data images into deconvolved images.

Parameter	Description
Display Mode	ApoTome images are acquired as raw data. The **Display Mode** sets how the image is calculated and displayed.
– Optical sectioning	The displayed output image is calculated/rendered by eliminating the excitation and emission light that originates in regions outside of the focal plane.
– Conventional fluorescence	The displayed output image is calculated/rendered like a conventional fluorescence image.
– Raw data	Displays the raw data as output image and disables all other parameters of the function.
Correction	Applies stripe artifact correction to the resulting image. It attempts to remove stripe artifacts which may be caused by bleaching of the sample during acquisition or by slight deviations in the grid phase position.
– No correction	No correction is applied to the image.
– Local Bleaching	Corrects the bleaching for each pixel individually (default setting). This is usually the best method.
– Global Bleaching	Corrects bleaching by means of global bleaching correction, which is applied equally to the entire image.
– Phase Errors	Corrects phase errors in the image without additional bleaching correction.
– Phase Errors and Global Bleaching	Corrects phase errors in the image with additional global bleaching.
– Phase Errors and Local Bleaching	Corrects phase errors in the image with additional local bleaching.
Fourier Filter	The Fourier filter attempts to remove residual stripes.
– Off	Uses no Fourier filter to remove stripes.
– Weak	Uses a weak Fourier filter to remove stripes.
– Medium	Uses a medium Fourier filter to remove stripes.
– Strong	Uses a strong Fourier filter to remove stripes.
Normalization	Here you can select how the gray/color values that exceed or fall short of the value range should be dealt with. If you use this method with **Direct Processing**, only the **Clip** method is available and preselected.

Parameter	Description
– Clip	Automatically sets the gray levels that exceed or fall short of the predefined gray value range to the lowest or highest gray value (black or white). The effect corresponds to underexposure or overexposure. In certain circumstances some information may therefore be lost.
– Automatic	Normalizes the gray values automatically to the available gray value range.

See also

Using Direct Processing [▸ 346]
ApoTome tab [▸ 905]

9.12.13.5 Attach PSF (Point Spread Function)

This method is only available for batch processing.

This method attaches a previously created PSF to the image file.

Parameter	Description
Channel	Selects the channel where the PSF should be attached.
PSF File	Selects the PSF-file created by the image processing function Create PSF.

9.12.13.6 Calculate Histogram

This method calculates a histogram distribution for selected measurement parameters of a measurement data table.

Parameters

Parameter	Description
Columns	Define the measurement parameters for classification by entering the column numbers freely, e.g. 1,3,5, or 1-6 or 1,3-7,8. Clicking on the ... button to open the **Select columns** dialog. Here the column names of the data can be activated or deactivated by clicking on the relevant checkbox.
Class Boundaries	Select here, how you want the class boundaries of the calculated histogram to be determined.
- >=,…,<	A numerical value falls into the histogram class if it is greater than or equal to the lower class boundary and less than the upper class boundary.
- >,…,=<	A numerical value falls into the histogram class if it is greater than the lower class boundary and less than or equal to the upper class boundary.
Automatic Classification	**Activated**: The class boundaries are calculated automatically from the data. The value range from the lowest to the highest data value is divided into as many classes of equal width as you have set in the **Class Number** input field.

Parameter	Description
	Example: Minimum value is 0 Maximum value is 10000 Range is 10000 units Class Count is 4 Then the class boundaries are as follows: Class 1: 0 .. 2500 Class 2: 2501 .. 5000 Class 3 : 5001 .. 7500 Class 4: 7501 .. 10000
Logarithmic	Only active, if the **Automatic Classification** checkbox is activated. **Activated**: The class boundaries are scaled logarithmic. Example: Minimum value is 0 Maximum value is 10000 Range is 10000 units Number of classes is 4 Then the class boundaries are as follows: Class 1: 0 .. 10 Class 2: 11 .. 100 Class 3: 101 .. 1000 Class 4: 1001 .. 10000
Class Count	Specifies the number of classes that shall be created.
Display Mode	Select here, how you want the values of the histogram to be calculated.
- Count	The histogram indicates how many data sets fall into the relevant class, it contains the frequency of the values in the class concerned.
- Count Cumulative	The histogram cumulates the counts of values in each class. Class 1 contains the number of values for class 1, class 2 contains the sum of the values from class 1 and class 2, class 3 contains the sum of the values from class 2 and class 3, etc.
- Percentage	The histogram indicates what percentage of the data sets fall into the relevant class, it therefore contains the percentage share of the values in the class concerned.
- Percentage Cumulative	The histogram cumulates the percentage of the count of values in each class. Class 1 contains the percentage for class 1, class 2 contains the sum of the percentages from class 1 and class 2, class 3 contains the sum of the percentages from class 2 and class 3, etc. The last class therefore contains 100%.
- Sum	The histogram contains the sum of the numerical values of the data sets that fall into the relevant class, the values of the data sets that fall into the class concerned are therefore added together.

Parameter	Description
- Sum Cumulative	The histogram cumulates the sums of the values in each class. Class 1 contains the sum of the numerical values from class 1, class 2 contains the sum of the numerical values from class 1 and class 2, class 3 contains the sum of the numerical values from class 2 and class 3, etc. The last class therefore contains the sum of all individual values.
- Percentage Sum	The histogram indicates the percentage share of the total numerical values in the relevant class.
- Percentage Sum Cumulative	The histogram cumulates the percentage of the sums of values of all data points which belong to the class. Class 1 contains the percentage of the total numerical values from class 1, class 2 contains the sum of the percentages of the total numerical values from class 1 and class 2, class 3 contains the sum of the percentages of the total numerical values from class 2 and class 3, etc. The last class therefore contains 100%.

9.12.13.7 Change Pixel Type

This method allows you to change the pixel type of an image. This can be useful if you want to compare or combine images that have different pixel types.

Parameters

Parameter	Description
Pixel Format	Select the desired pixel format from the dropdown list.
- 8 Bit B/W	The output image is a monochrome image, the whole-number gray values of which can lie in the range from 0 to 255.
- 16 Bit B/W	The output image is a monochrome image, the whole-number gray values of which can lie in the range from 0 to 65535.
- 32 Bit B/W Float	The output image is a monochrome image with real numbers as pixel values.
- 2x32 Bit Complex	The output image is a monochrome image with complex numbers (real part and imaginary part) as pixel values. Such images are generally created by means of transformation into the Fourier space.
- 24 Bit RGB	The output image is a color image, the whole-number color values of which in the red, green and blue channels can lie in the range from 0 to 255.
- 48 Bit RGB	The output image is a color image, the whole-number color values of which in the red, green and blue channels can lie in the range from 0 to 65535.
- 2x32 Bit RGB Float	The output image is a color image with real numbers as color values in the red, green and blue channels.
- 3x64 Bit RGB Complex	The output image is a color image with complex numbers (real part and imaginary part) in the red, green and blue channels. Such images are generally created by means of transformation into the Fourier space.

9.12.13.8 Combine HLS

With this method a HLS image can be generated of the single color extractions H, L, S.

9.12.13.9 Combine RGB

With this method a color image can be generated out of three input images of the single color extractions **Red**, **Green** and **Blue**.

Parameters

Parameter	Description
Output Pixel type	Here you choose the desired output image format, e.g. 24 Bit RGB.

9.12.13.10 Convert To Lambda

With this function you can convert Lambda stacks which were acquired with **LSM 800** into a file with the same appearance as inside the **Lambda** view. In contrast to the generic raw data format of the Lambda stacks, these files can be opened and analyzed normally in **ZEN (black edition)**.

9.12.13.11 Copy Annotations

This method copies the annotations of one image into another image.

9.12.13.12 Copy Image

This method creates a copy of an image.

9.12.13.13 Correct Stage Jitter

This method automatically corrects the jitter of the stage which can occur during the acquisition of a Z-stack image.

9.12.13.14 Correlation

With this function you can, in conjunction with confocal data sets, display the spatial or temporal correlation of an image or image stack. You can select which kind of correlation you want to perform by activating the corresponding checkboxes.

Parameters

Parameter	Description
Cross Correlation	If activated, you can to correlate two images with each other. Note that the second input image needs to have the same dimensionality and size.
X, Y	Correlates the signal in the X or Y direction.
Z	Correlates the signal in Z. Only available for data sets containing Z-sections.

Parameter	Description
Time	Correlates the signal in time. Only available for time series data sets.

9.12.13.15 Create Gray Scale Image

This method allows you to create a gray scale image.

Parameters

Parameter	Description
Pattern	Select the desired pattern for the gray scale image here.
- Uniform	All pixels have an identical gray/color value.
- 2D Gray Scale Vertical	The gray scale runs from top to bottom, starting with the gray value selected in parameter **Min. Gray Value**.
- 2D Gray Scale Horizontal	The gray scale runs from left to right, starting with the gray value selected in parameter **Min. Gray Value**.
Width	Set the desired width of the output image in pixels using the slider or the input field.
Height	Set the desired height of the output image in pixels using the slider or the input field.
Min. Gray Value	Set the minimum gray value of the gray scale using the slider or input field.
Max. Gray Value	Set the maximum gray value of the gray scale using the slider or input field.
Pixel Type	Select the desired pixel type here.
- 8 Bit B/W	The output image is a monochrome image whose integer gray values can be in the range of 0 to 255.
- 16 Bit B/W	The output image is a monochrome image whose integer gray values can be in the range of 0 to 65535.
- 24 Bit RGB	The output image is a color image whose integer color values in the channels Red, Green, Blue can be in the range of 0 to 255.
- 48 Bit RGB	The output image is a color image, with integer color values in the color channels Red, Green, Blue can be in the range of 0 to 65535.

9.12.13.16 Create Image Subset

This method allows you to extract parts from one image and use these to create a new image. You can select these parts freely from the individual dimensions of the image.

Info

Each of the sections described below is only visible if the corresponding dimension is present in the input image.

Parameter	Description
Channels	Here you can select which channels of the input image you want to be used. All channels are selected by default. To deselect a channel, click on the respective channel button.
Z-Position, **Time**, **Block**, **Scene**	Here you can select which parts of the input image you want to use for the resulting image.
- Extract All	If selected, all parts of the corresponding image are extracted.
- Extract Single	If selected, you can select a single image to be extracted.
- Extract Range	If selected, you can select a certain range of images to be extracted.
- Extract Multiple	If selected, you can select several continuous ranges and individual sections. Enter one or more sections that you want to select in the input field. To do this, enter the first section, followed by a minus sign, and then the last section. If you want to define an interval, after the last section enter a colon and then the interval. The entry "2-10:2" means that every second section is selected from section 2 to section 10. Enter a comma after the first section if you want to define another section. You can also select individual sections separated by commas. By entering "2-10:2,14-18,20,23", you select every second section from section 2 to section 10, followed by sections 14 to 18, as well as sections 20 and 23.
- Get current position	Adopts the position from the current display in the image area.
- Interval	**Activated:** Interval mode is active. The Interval spin box/input field appears. Enter the desired interval here. E.g. if you enter the value 2 only every 2nd value from the range is considered.
Region	Here you can select if you want to use the entire image or just a region (ROI) of the input image.
- Full	Select this option to use the full image for the new image.
- Rectangle region (ROI)	Select this option to draw in a rectangle region of interest which will be used for creating a new image. If a rectangle region was drawn in you can see and change its coordinates by editing the **X/Y/W/H** input fields.
- Keep tiles	Has only an effect, if a region (ROI) is defined. **Activated:** Extracts the drawn in region including the complete tiles. This setting is recommend when you want to apply DCV processing functions on the resulting image.

9.12.13.17 Create Image Subset and Split

This method allows you to extract certain dimensions, e.g. channels, regions or time series from one image and use these to create a new image.

Info

Each of the dimensions described below is only visible if the corresponding dimension is present in the input image.

Method Parameters

Parameter	Description
Split Dimension	Depends on the loaded image.
- None	The image is not split by any dimension. Only the ranges of the different dimensions defined below will be extracted for the new image.
- Channels (or: Time, Scenes etc.)	Here you can select the dimension for splitting the dataset. A new image document opens in ZEN for each element of the selected dimension. The available options depend on the selected image. If your input image contains two channels, split dimension creates two output images, for each channel one.
Channels	Here you can select which channels of the input image you want to be used. All channels are selected by default. To deselect a channel, click on the respective channel button.
Time (or: Z-Position, Rotation, Scene, Illumination, Acquisition block, Mosaic tile, Phase, View)	
- Extract All	**Activated**: All elements of the corresponding dimension are extracted.
- Extract Single	**Activated**: You can select a single element to be extracted.
- Extract Range	**Activated**: You can select a certain range of elements to be extracted.
- Extract Multiple	**Activated**: You can select several continuous ranges and individual sections. Enter one or more sections that you want to select in the input field. To do this, enter the first section, followed by a minus sign, and then the last section. If you want to define an interval, after the last section enter a colon and then the interval. The entry "2-10:2" means that every second section is selected from section 2 to section 10. Enter a comma after the first section if you want to define another section. You can also select individual sections separated by commas. By entering "2-10:2,14-18,20,23", you select every second section from section 2 to section10, followed by sections 14 to 18, as well as sections 20 and 23.

Parameter	Description
Region	
- Full	Takes the full region into account.
- Rectangle Region	Takes the rectangle into account that you can draw in the 2D view. After drawing you can modify X, Y coordinates as well as width (W) and height (H) manually.
Keep tiles	Has only an effect, if a region (ROI) is defined. **Activated**: Extracts the drawn in region including the complete tiles. This setting is recommend when you want to apply DCV processing functions on the resulting image. **Deactivated**: Drawn ROI will cut through mosaic tiles.
Propagate ROI	Has only an effect, if a region (ROI) is defined in multi-scene images. **Activated**: Applies the defined region (ROI) to all scenes.

For more information, see *Creating image subset and split dimensions [▸ 107]*

9.12.13.18 Create Image Subset and Split (Write files)

This method allows you to extract certain dimensions, e.g. channels, regions or time series from one image and use these extracted dimensions to create a new image. The result file is saved in your target folder.

Info

Each of the dimensions described below is only visible if the corresponding dimension is present in the input image.

Method Parameters

Parameter	Description
Split Dimension	Depends on the loaded image.
- None	The image is not split by any dimension. Only the ranges of the different dimensions defined below will be extracted for the new image.
- Channels (or: Time, Scenes etc.)	Here you can select the dimension for splitting the dataset. A new file will be created in the target folder for each element of the selected dimension. The available options depend on the selected image. If your input image contains two channels, split dimension creates two output images for each channel.
Channels	Here you can select which channels of the input image you want to be used. All channels are selected by default. To deselect a channel, click on the respective channel button.
Time (or: Z-Position, Rotation, Scene, Illumination, Acquisition block, Mosaic tile, Phase, View)	

Parameter	Description
- Extract All	**Activated**: All elements of the corresponding dimension are extracted.
- Extract Single	**Activated**: You can select a single element to be extracted.
- Extract Range	**Activated**: You can select a certain range of elements to be extracted.
- Extract Multiple	**Activated**: You can select several continuous ranges and individual sections. Enter one or more sections that you want to select in the input field. To do this, enter the first section, followed by a minus sign, and then the last section. If you want to define an interval, after the last section enter a colon and then the interval. The entry "2-10:2" means that every second section is selected from section 2 to section 10. Enter a comma after the first section if you want to define another section. You can also select individual sections separated by commas. By entering "2-10:2,14-18,20,23", you select every second section from section 2 to section10, followed by sections 14 to 18, as well as sections 20 and 23.
Region	
- Full	Takes the full region into account.
- Rectangle Region	Takes the rectangle into account that you can draw in the 2D view. After drawing you can modify X, Y coordinates as well as width (W) and height (H) manually.
Keep tiles	Has only an effect, if a region (ROI) is defined. **Activated**: Extracts the drawn in region including the complete tiles. This setting is recommend when you want to apply DCV processing functions on the resulting image. **Deactivated**: Drawn ROI will cut through mosaic tiles.
Propagate ROI	Has only an effect, if a region (ROI) is defined in multi-scene images. **Activated**: Applies the defined region (ROI) to all scenes.
Target Folder	Selects the folder on the disk where the images are to be saved.
Overwrite existing files	Overwrites image files.
Compression	
- Original	The output image has the same compression as the original image.
- Compression	JPEG XR compession is applied to the output images.
Defaults	Sets the values back to default, if they have been changed.

For more information, see *Creating image subset and split dimensions [▸ 107]*

9.12.13.19 Create PSF

For creating experimental point spread functions from a Z-stack of subresolution fluorescent beads please use the function **PSF Wizard** which is available together with the **Deconvolution** module and offers a guided procedure starting with a stack of many beads and includes the **Create PSF** functionality.

Prerequisite for the **Create PSF** function here is, that bead averaging has already been done. It is available only for legacy reasons.

This function creates a PSF (Point Spread Function) image from a Z-stack image of a bead acquired for PSF measurement. Please observe the instructions for optimal acquisition here: Using beads for PSF measurement.

The result is a so-called PSF image. For advanced settings and options, please use the specific control elements on the *PSF Display [904]* tab.

Parameters

Parameter	Description
Z-Stack Correction	**Activated:** Performs background correction of the Z-stack before the processing.
Circular Average	**Activated:**Forces a PSF with lateral symmetry. This option should not usually be activated as lateral asymmetries correspond better to the real situation. Circular averaging is only recommended when a measured PSF is used with the Fast Iterative method.
Threshold Cropping	**Activated:** The PSF is restricted to gray value ranges up to 0.25% of the brightest voxel present. If the value is reduced or the option is deactivated, the PSF may be larger. This increases the calculation time. However, it is also possible to achieve slightly better results in this case. This option is deactivated by default.
Threshold	Using this slider and input field you can set the percentage from which the PSF is clipped if the Volume Clipping option has been selected.
Iterative Restoration	**Activated:** If Z-stack images of beads with diameters greater than the microscope's resolution limit are used to generate the PSF, this option must be selected. The bead diameter used can be entered using the slider and input field.

9.12.13.20 Fuse Image Subset

This method allows you to insert an image subset back into the original image. Its contents are replaced by the contents of the image subset. Using this method you can process a previously created image subset using image processing functions and copy the result back into the original image.

Parameters

Parameter	Description
In Place	

Parameter	Description
Subset	Contains the description of how the input image was created as a subset. Shows which areas have been selected in generating the subset image for each dimension (channels, Z-stack, time series), as well as for the defined image section. **Example:** The entry "Z (1-8: 2) | T (2-7)" means that the sub-image consists of sections 1,3,5,7 at the intervals of 2 to 7 of the input image.

9.12.13.21 Generate Image Pyramid

This method allows you to calculate a resolution pyramid for an image. Using the resolution pyramid you can navigate extremely efficiently even in very large tile images and display individual regions in the image window.

Parameters

Option	Description
Background	This option will influence how the background of the image pyramid will look like.
– Auto	The region outside the scenes keeps the grey value of the image, i.e. white for brightfield images and black for fluorescence images.
– Black	The region outside the scenes will be displayed black.
– White	The region outside the scenes will be displayed white.

9.12.13.22 Image Calculator

This method allows you to apply arithmetic operations to images in the form of a calculator.

You can process a single image or combine two images.

All operations are performed pixel by pixel.

Parameters

Parameter	Description
Channel Input 1	Here you can select whether you want to use an individual channel or all channels of the first input image for the calculation.
Channel Input 2	Here you can select whether you want to use an individual channel or all channels of the second input image for the calculation.
First Images	**Activated:** For the second input image uses only the first time points of a time lapse image for the calculation. This allows you, for example, to normalize a time lapse image to the intensity values of the first time points. Enter the number of images that you want to be used for the calculation using the input field.

Parameter	Description
Formula	Enter the calculation formula here using the keyboard and numeric keypad. Use "S1" as a placeholder for the first input image and "S2" for the second input image.
Input 1	Inserts the placeholder for the first input image into the **Formula** input field at the current cursor position.
Input 2	Inserts the placeholder for the second input image into the **Formula** input field at the current cursor position.
Absolute Intensities	Activate this radio button if input image 1 and input image 2 have the same pixel type.
Normalize Intensities 0..1	Activate this radio button if input image 1 and input image 2 have different pixel types. To allow such images to be combined, the intensity values of the two images are normalized to the value range from 0 to 1 before the calculation.
Operators...	Opens a list of all available operators. Here you can select the operator that you want. If you double-click on a list entry, it is inserted into the **Formula** input field at the current cursor position.
Delete	Deletes the contents of the **Formula** input field.
Undo	Undoes the last entry in the **Formula** input field.

9.12.13.23 Image Generator

This function creates a synthetic image where the dimensions can be defined.

Parameter	Description
Width	Width in x of the image
Height	Height in y of the image
Z Slices	Number of z slices of the image. If the value is > 1, it will become a Z-stack image.
Channels	number of channels of the image, if value is > 1, it will become a multi channel image
Time Slices	Number of time slices, if value is > 1, it will become a time series image.
Min. Gray Value	Minimum Gray Value for generation.
Max Gray Value	Maximum Gray Value for generation.
Pixel Type	Specifies the pixel type of the image.
Pattern	
- Uniform	all pixels of the image have identical Min. Gray Value
- 2D Gray Scale Vertical	the image shows a gray scale with values between **Min. Gray Value** and **Max. Gray Value** from top to bottom.
- 2D Gray Scale Horizontal	the image shows a gray scale with values between **Min. Gray Value** and **Max. Gray Value** from left to right.

Parameter	Description
- Ramp	the image shows a ramp with values between **Min. Gray Value** and **Max. Gray Value** starting from each corner of the image to the center.
- Gaussian	the image shows a Gaussian shaped grayscale with values between **Min. Gray Value** and **Max. Gray Value** starting from the borders of the image to the center.
- Checkerboard	the image shows a checkerboard where the “dark” fields have **Min. Gray Value** and the “bright” fields have **Max. Gray Value**.
- Cosine Checkerboard	the image shows a checkerboard where the “dark” fields have **Min. Gray Value** and the “bright” fields have **Max. Gray Value** overlaid with a cosine modulation.
- Chirp Cosine	the image shows a cosine pattern where the “dark” fields have **Min. Gray Value** and the “bright” fields have **Max. Gray Value** overlaid with a chirp modulation.
- Chirp Checker	the image shows a checkerboard where the “dark” fields have **Min. Gray Value** and the “bright” fields have **Max. Gray Value** overlaid with a chirp modulation.
- Random Spheres	a 3D (Z stack) image is created which contains **Number of Spheres** spheres with **Sphere Diameter** diameter which are randomly distributed in the image.
- Sphere Array	a 3D (Z stack) image is created which contains **Number of Spheres** spheres with **Sphere Diameter** diameter which are equally distributed in the image.
- Single Sphere	a 3D (Z stack) image is created which contains a single sphere with **Sphere Diameter** diameter which is positioned in the center of the image.
Sphere Diameter	Diameter of the created spheres.
Number of Spheres	Number of spheres which are generated in the 3D image.

9.12.13.24 Impose Noise

This function imposes an image with a defined noise for testing purposes.

Parameter	Description
Signal to Noise Ratio	Adjusts the signal to noise ratio. Range 0.10 - 100.00.
Distribution	
- Poisson	Imposes a Poisson distributed noise.
- Gauss	Imposes a Gauss distributed noise.

9.12.13.25 Linear Unmixing

With this function you can extract the emission of single fluorescence dyes (e.g. GFP only, YFP only etc.) from strongly overlapping multi-fluorescence data acquired in multi-channel images or Lambda stacks (only available in LSM imaging mode).

With the knowledge of the spectral characteristic of individual components within a multi-component sample, even heavily overlapping individual spectral characteristics can be mathematically extracted from an experimental multi-channel data set. This method is a strictly pixel-by-pixel image analysis procedure. Experimentally, fluorescence spectra of mono-labeled samples are acquired and stored in the Spectra Database as an external reference. Then a multi-channel image or Lambda stack from the multi-labeled sample is acquired. The individual components are mathematically extracted using the information from the reference spectra. Up to ten different reference signals can be used in the least-square-fit based algorithm to produce a 10-channel output image without any partial overlap between the channels.

Avoid saturation of fluorescence signal in the data set to be unmixed. Saturation will generate a high signal in the residual channel.

If mono-labeled samples are not available, the references can be obtained by the following methods:

- Interactively by user-selection of regions in the image where only one fluorescence dye is present (only available in the **Unmixing** view).
- Automatically by software analyses of what the individual spectral signatures are. This processing function is called Automatic Component Extraction (ACE).

Note that in some cases, spectrally acquired images are not appropriate for ACE and linear unmixing can lead to wrong results.

Parameters

Parameter	Description
Components	Adjust the number of spectrally distinguishable fluorescent components within the imaged sample. The number of extractable components cannot be higher than the number of acquired channels. The maximum possible value is 10 components.
Import Reference Spectra	For the unmixing process previously generated emission spectra of ideally pure dyes can be loaded and used for unmixing. This function is mutually exclusive to the **Automatic Component Extraction** function.
Automatic Component Extraction	Use this function if no reference spectra are available. Indicate the number of components the system should be looking for in the image. The number of components cannot be higher than the number of channels. It will only work, if each of the emission signals is present in an area of the image without overlap of another emission signal. Otherwise **ACE** cannot produce a reliable result.
Weighted Unmixing	When this option is checked, spectral channels with high noise contribute less to the unmixing result. This option includes a statistical analysis of the signal-related (Poisson-) noise and weighs the respective contribution for the fitting with the combination of reference spectra to the experimental data.

Parameter	Description
	Note: This option involves a more sophisticated unmixing algorithm and therefore takes longer than the basic unmixing analysis. Weighted unmixing generates improved unmixing results when acquisition channels are not so well balanced but still have a good signal-to-noise ratio.
Autoscale	Balances the intensity of the unmixed channels to equal levels.
Calculate Residuals	Generates an additional channel in which the intensity values represent the difference between the acquired spectral data and the fitted linear combination of the reference spectra. In essence, the residual value is the biggest remaining "residual" from the least square fit routine. The residuals are a general measure for how good the fit of the algorithm has performed. The higher the intensity in this additional channel, the worse is the fit of the spectra to the data set.

9.12.13.26 Split into HLS

This method generates the individual color extractions for a HLS input image. The resulting images for hue, lightness and saturation take the form of gray images.

9.12.13.27 Split into RGB

This method generates the individual color extractions for red, green and blue from the RGB input image. The resulting images for red, green and blue take the form of gray images.

Parameters

Parameter	Description
Output Pixel type	Here you choose the desired output image format, e.g. 8 Bit B/W.

9.12.13.28 Split Multiblock Image

This method saves the single blocks/dimensions (Tiles or Positions) of a multiblock image (i.e. image of an inhomogeneous experiment) in a folder in **.CZI** format.

Parameter

Parameter	Description
Split Mode	Choose the mode how to split the multiblock image.
- Homogeneous groups	Splits the multiblock image into the single dimensions. The blocks will remain.
- Single blocks	Splits the multiblock image into single blocks.
Display field	The path of the destination folder is displayed automatically in the display field. To change the folder, click on the ... button to the right of the display field.

9.12.13.29 Split Scenes

This method separates scenes from a tiles or positions image. The individual images are displayed in the **Center Screen Area**. Note that the images in this method, in contrast to the method **Split Scenes (write files)**, are not automatically stored in a folder.

9.12.13.30 Split Scenes (Write files)

This method saves the single scenes (tiles or positions) of a multiscene image (i.e. image of a multiwell plate) as single images in a folder in **CZI** format.

Parameter

Parameter	Description
Display field	The path of the destination folder is displayed automatically in the display field. To change the folder, click on the ... button to the right of the display field.
Include Scene Information in Generated File Name	**Activated:** Includes the scene information in the file name of the separate image.
Overwrite existing files	**Activated:** All files in the target folder will be deleted, if the function is applied again.
Compression	Choose the type of compression here, eg. **Original** (no compression) or **Compression (JPEG XR)**.

9.12.13.31 VivaTome RAW Convert

9.12.14 Export/Import

9.12.14.1 Movie Export

Using this function you can export multidimensional images (e.g. Time Series or Z-Stack images) into various file types in the form of film sequences so that you can continue to use them in other programs.

Info

If you want to export **MOV** files (H264 or MPEG4 codec) successfully, download the application **FFmpeg Version 4.0.2, Windows 64-bit, Static** (e.g. on http://www.ffmpeg.org/ or https://ffmpeg.zeranoe.com/builds/). Copy **ffmpeg.exe** in to the same folder where **ZEN.exe** is located (for example C:\Program Files\Carl Zeiss\ZEN 2\ZEN 2 (blue edition)).

See also

Exporting Movies [233]

9.12.14.1.1 File Type section

Info

AVI (MS-Video1) mode is available for 32-bit Windows operating systems only.

Parameter	Description
Format	Select the desired mode here. The following formats are available for the movie export. ▪ AVI (M-JPEG compression) ▪ AVI (uncompressed) ▪ AVI (DV) ▪ WMF (WindowsMedia) ▪ MOV (H.264) ▪ MOV (MPEG4) ▪ AVI (MS-Video1)
Size/Rate	Select the desired format here.
- Original Size	Not available for the file types **AVI (DV)** and **AVI (MS-Video1)**. Uses the height and width of the input image and sets the frame rate to 5 frames per second.
- User-Defined	Not available for the file types **AVI (DV)** and **AVI (MS-Video1)**. Enter the values in the Width, Height and Frame Rate input fields.
- 720x576/25fps (PAL 576p/25)	Uses the PAL (Phase Alternating Line) video resolution with 25 frames per second.
- 720x480/29.97fps (NTSC)	Uses the NTSC (National Television Systems Committee) video resolution with 29.97 frames per second.
- 1280x720/50fps (HD 720p/50)	Not available for the file types **AVI (DV)** and **AVI (MS-Video1)**. Uses the HD (High Definition 720) video resolution with 25 frames per second.
- 1920x1080/25fps (HD 1080p/25)	Not available for the file types **AVI (DV)** and **AVI (MS-Video1)**. Uses the HD (High Definition 1080) video resolution with 25 frames per second.
- 1920x1080/29.97fps (HD 1080p/29.97)	Not available for the file types **AVI (DV)** and **AVI (MS-Video1)**. Uses the HD (High Definition 1080) video resolution with 29.97 frames per second.
Width	Only active if you have selected **User-Defined** in the **Format** drop-down list. Here you can enter the width of the image in pixels (px).
Height	Only active if you have selected **User-Defined** in the **Format** drop-down list. Here you can enter the height of the image in pixels (px).

Parameter	Description
Frame Rate	Only active if you have selected **User Defined** in the **Format** drop-down list. Here you can enter the frame rate in frames per second (fps).
Quality	Only visible if you have selected **AVI (M-JPEG compression)** or **MOV** in the **Mode** dropdown list. Here you can set the image quality using the slider or spin box/input field. This influences the size of the file. Although low values result in very small files, image quality may be considerably reduced.

9.12.14.1.2 Image Data section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Apply Display Settings and Channel Color	
– **Burn-in Graphics**	**Activated:** Burns the graphic elements into the image. The pixels under the graphic element (e.g. scale bars) are overwritten. The burnt-in graphic elements cannot be subsequently modified.
– **Zoom**	Only available if **Burn-in Graphics** is activated. Sets the zoom of graphic elements for export. The default is 100%, which means that the graphical elements are rendered as they would if the viewer zoom was 100%. When the zoom increases, the graphical elements are rendered larger (e.g. with zoom 200, graphical elements are rendered twice as large, but it corresponds to the size they would have if the viewer zoom was 50%).
– **Get Zoom from Viewer**	Only available if **Burn-in Graphics** is activated. Gets the current zoom factor of the graphical elements in the 2D view.
– **Merged Channels Image**	Only visible for multichannel images. **Activated:** Exports the pseudo color image of all selected channels.
– **Individual Channels Image**	Only visible for multichannel images. **Activated**: Exports the individual colored image of all selected channels.
Use channel names	Only visible for multi-channel images. **Activated:** Integrates the channel name in the name of the exported image.
Use Full Set of Dimensions	**Activated:** Includes all dimensions of the original file.
Define Subset	**Activated:** Creates a subset of the data, depends on the dimensions available in the image, e.g. Channels, Region, Tiles.

At least one of the three checkboxes must be activated. If the **Merged Channels Image** and **Individual Channel Image** checkboxes are activated, you can export the individual colored images and the pseudo color images in a single step.

9.12.14.1.3 Fitting section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Fitting	Select the desired type of fitting here.
- Fit All (Uniform)	Fits the image to the selected resolution. The original aspect ratio is retained.
- Fit and Crop (Uniform to Fill)	Fits the image to the selected resolution and clips it. The original aspect ratio is not retained.
- Fit and Stretch (Fill)	Stretches the image to the selected resolution. The original aspect ratio is not retained.
- Crop (None)	Crops the image to the selected resolution. The original aspect ratio is retained.

9.12.14.1.4 Sequence section

The following functions are only visible if the **Show All** mode is activated:

Change the sequence of the dimensions in which you want the movie to be created.

button

Shifts the selected dimension up a line.

button

Shifts the selected dimension down a line.

9.12.14.1.5 Mapping section

Parameter	Description
Mapping	Select how you want the images to be assigned.
- Fixed Duration	Enter the time per image in seconds using the spin box/input field. The total length is displayed in the **Final Movie Length** display field.
- 1 Frame per Image	Assigns one frame per image.
Image count	Indicates the number of images in the input image.
Final Movie Length	Indicates the total length of the resulting movie, depending on the selected image sequence and the time.

9.12.14.1.6 Dimensions section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Use Full Set of Dimensions	Select this option if you want to export all dimensions without changing them.
Define Subset	Select this option if you only want to export individual dimensions or subsets of individual dimensions.

Info

Each of the sections described below is only visible if the corresponding dimension is present in the input image.

Parameter	Description
Channels	Here you can select which channels of the input image you want to be used. All channels are selected by default. To deselect a channel, click on the relevant channel button.
Z-Position, **Time**, **Block**, **Scene**	Here you can select which parts of the input image you want to use for the resulting image.
- Extract All	If selected, all parts of the corresponding image are extracted.
- Extract Single	If selected, you can select a single image to be extracted.
- Extract Range	If selected, you can select a certain range of images to be extracted.
- Extract Multiple	If selected, you can select several continuous ranges and individual sections. Enter one or more sections that you want to select in the input field. To do this, enter the first section, followed by a minus sign, and then the last section. If you want to define an interval, after the last section enter a colon and then the interval. The entry "2-10:2" means that every second section is selected from section 2 to section 10. Enter a comma after the first section if you want to define another section. You can also select individual sections separated by commas. By entering "2-10:2,14-18,20,23", you select every second section from section 2 to section 10, followed by sections 14 to 18, as well as sections 20 and 23.
- Get current position	Adopts the position from the current display in the image area.
- Interval	**Activated:** Interval mode is active. The Interval spin box/input field appears. Enter the desired interval here. E.g. if you enter the value 2 only every 2nd value from the range is considered.
Region	Here you can select if you want to use the entire image or just a region (ROI) of the input image.
- Full	If selected this option, the full image is used for the new image.

Parameter	Description
- Rectangle region (ROI)	If selected this option, you can draw in a rectangle region of interest which will be used for creating a new image. If a rectangle region was drawn in you can see and change its coordinates by editing the **X/Y/W/H** input fields.
- Keep tiles	Has only an effect, if a region (ROI) is defined. **Activated:** Extracts the drawn in region including the complete tiles. This setting is recommend when you want to apply DCV processing functions on the resulting image.
Tiles	
- Existing Tiles	Exports the tiles according to the original image.
- Re-Tile	Creates new tiles according to your entries in the fields **Colums/Rows** and **Overlap**.
- Colums /Rows	Only active if **Re-Tile** is activated. Defines, with how many rows and colums the re-tiling is performed.
- Overlap	Only active if **Re-Tile** is activated. Defines the overlap of the re-tiled tiles in %.

9.12.14.1.7 Export to section

Parameter	Description
Export to	The path of the export folder is displayed automatically in the display field. To change the file path, click on the ... button to the right of the display field.

Parameter	Description
Prefix	Here you can edit the prefix specified or enter a new name. The name of the original image is specified by default.

9.12.14.2 OME TIFF Export

Using the **OME TIFF Export** function you can export your images into OME (Open Microscopy Environment) TIFF format so that you can continue to use them in other programs. The images are then available as a multipage TIFF file.

9.12.14.2.1 Image Format section

Parameter	Description
Resize	Adjust the image size in percent using the slider or spin box/input field.

Parameter	Description
The following parameters are only visible, if **Show All** is activated.	
BigTIFF	**Activated:** Creates a BigTIFF image that can be bigger than 4 gigabytes and uses 64-bit offset format.
Compress	**Activated:** Performs internal tif compression.
Use Tiles	**Activated:** Performs an internal tiling. Note that this option is recommend for large images as some tif readers do not support large images.
Shift Pixel	**Activated:** Shifts the grey value of a 10-bit or 12-bit image to 16-bit.
Merge All Scenes	**Activated:** Generates one image including all scenes. Single scene images will be generated, if the checkbox is deactivated.

9.12.14.2.2 Image Data section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Original Data	**Activated:** Exports the image with the original channel colors and the original display characteristic curve.
Apply Display Curve and Channel Color	**Activated:** Exports the image with the changed channel color and display characteristic curve settings. These settings are applied to the pixel values of the exported images. They are particularly important if you want to use dark images with a dynamic range of more than 8 bits in other programs.
Burn In Graphics	**Activated:** Burns the graphic elements into the image. The pixels under the graphic element (e.g. scale bars) are overwritten. The burnt-in graphic elements cannot be subsequently modified.

9.12.14.2.3 Dimensions section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Use Full Set of Dimensions	Select this option if you want to export all dimensions without changing them.
Define Subset	Select this option if you only want to export individual dimensions or subsets of individual dimensions.

Info

Each of the sections described below is only visible if the corresponding dimension is present in the input image.

Parameter	Description
Channels	Here you can select which channels of the input image you want to be used. All channels are selected by default. To deselect a channel, click on the relevant channel button.
Z-Position, **Time**, **Block**, **Scene**	Here you can select which parts of the input image you want to use for the resulting image.
- Extract All	If selected, all parts of the corresponding image are extracted.
- Extract Single	If selected, you can select a single image to be extracted.
- Extract Range	If selected, you can select a certain range of images to be extracted.
- Extract Multiple	If selected, you can select several continuous ranges and individual sections. Enter one or more sections that you want to select in the input field. To do this, enter the first section, followed by a minus sign, and then the last section. If you want to define an interval, after the last section enter a colon and then the interval. The entry "2-10:2" means that every second section is selected from section 2 to section 10. Enter a comma after the first section if you want to define another section. You can also select individual sections separated by commas. By entering "2-10:2,14-18,20,23", you select every second section from section 2 to section 10, followed by sections 14 to 18, as well as sections 20 and 23.
- Get current position	Adopts the position from the current display in the image area.
- Interval	**Activated:** Interval mode is active. The Interval spin box/input field appears. Enter the desired interval here. E.g. if you enter the value 2 only every 2nd value from the range is considered.
Region	Here you can select if you want to use the entire image or just a region (ROI) of the input image.
- Full	If selected this option, the full image is used for the new image.
- Rectangle region (ROI)	If selected this option, you can draw in a rectangle region of interest which will be used for creating a new image. If a rectangle region was drawn in you can see and change its coordinates by editing the **X/Y/W/H** input fields.
- Keep tiles	Has only an effect, if a region (ROI) is defined. **Activated:** Extracts the drawn in region including the complete tiles. This setting is recommend when you want to apply DCV processing functions on the resulting image.
Tiles	
- Existing Tiles	Exports the tiles according to the original image.
- Re-Tile	Creates new tiles according to your entries in the fields **Colums/Rows** and **Overlap**.

Parameter	Description
- Colums /Rows	Only active if **Re-Tile** is activated. Defines, with how many rows and colums the re-tiling is performed.
- Overlap	Only active if **Re-Tile** is activated. Defines the overlap of the re-tiled tiles in %.

9.12.14.2.4 Export to section

Parameter	Description
Export to	The path of the export folder is displayed automatically in the display field. To change the file path, click on the ... button to the right of the display field.

Parameter	Description
Prefix	Here you can edit the prefix specified or enter a new name. The name of the original image is specified by default.

9.12.14.3 ZVI Export

Using the **ZVI Export** function you can export your images into ZVI format so that you can continue to use them in AxioVision.

9.12.14.3.1 Export to section

Parameter	Description
Export to	The path of the export folder is displayed automatically in the display field. To change the file path, click on the ... button to the right of the display field.

Parameter	Description
Prefix	Here you can edit the prefix specified or enter a new name. The name of the original image is specified by default.

9.12.14.4 Image Import

Using the **Image Import** function you can create a multidimensional image (multichannel, Z-stack, time lapse, tile, position image) from individual images. The individual images may be in any of the external formats supported by ZEN (see below). The resulting image can then be saved in **CZI** format and processed further using the functions available in ZEN.

Supported file types

- JPG images
- BMP
- TIFF
- PNG
- GIF
- DeltaVision images
- MetaFluor images
- Multi page images

Parameters

Parameter	Description
Multichannel	**Activated:** Activates the settings to import multichannel images. Adjust the settings for the multichannel image to be imported in the list below.
- Use Channel Name as Identifier	**Activated:** Uses the name specified in the **Name** column to identify the channel. The channel name will appear in the **Preview** display field in the **Specify the Identifiers** section. **Deactivated:** Activates the **Identifier**, **Start Index** and **Interval** columns in the **Specify the Identifiers** section.
Z-Stack	**Activated:** Activates the settings to import Z-Stack images. Adjust the settings for the Z-stack image to be imported in the list below.
- Interval	Here you enter the value in µm for the distance between the individual slices. The total height of the Z-stack is calculated automatically from this value and the number of slices.
- Slices	Here you enter the number of slices of the Z-Stack image.
- Range	Here you enter the total height (range) of the Z-stack in µm. The distance between the individual Z-stacks is calculated automatically from this value and the number of slices and displayed in the **Interval** display field.

Parameter	Description
- Extended Microscope Parameters	Activates additional parameters that are necessary for further processing of the imported image (e.g. for deconvolution), like: ▪ **Magnification:** Select the objective magnification that was used for acquisition here. ▪ **Aperture:** Here you can enter the value of the numerical aperture of the objective that was used for acquisition. ▪ **Immersion:** Select the immersion medium that was used for acquisition here.
Time Series	**Activated:** Activates the settings to import time series images.
- Interval	Here you enter the value for the interval between the individual time points. Select the unit of time from the dropdown list to the right of the input field.
- Cycles	Here you enter the cycles of the time series. The entered value will affect the duration/the interval depending on which value you have selected.
- Duration	Here you can enter the value for the duration of the entire time series. Select the unit of time from the dropdown list to the right of the input field. Enter the number of time points in the **Time Points** input field.
Tiles	**Activated:** Activates the settings to import tile images.
- Columns	Here you can enter the number of columns of the tile image.
- Overlap	Here you can enter the percentage by which the tiles do overlap.
- Rows	Here you can enter the number of rows of the tile image.
- Meander	Select this option, if the images to be imported were acquired in the Meander acquisition/travel mode.
- Comb	Select this option if the images to be imported were acquired in the Comb acquisition/travel mode.
Positions	**Activated:** Activates the settings to import images of individual positions.
Scalings	To show the section in full, click on the arrow button
- Use Current Scaling	Uses the geometric scaling currently selected and displays the values for **Scale (X)** and **Scale (Y)** with the corresponding unit in the relevant display field.
- Define scaling	Enter the desired values in the **Scale (X)** input field and in the **Scale (Y)** spin box/input field. Select the unit for the scaling value from the dropdown list to the right of each input field.

Parameter	Description
Automatic	If selected, all images that are available in an import folder are imported automatically.
- Import From	Here you can select the import folder. To select a folder, click on the folder button to the right of the display field. The names of the images are displayed in the **File Name** list below the display field.
Sequential	If selected, an image sequence in a certain order (e.g. image dimensions that are encoded by means of numbers in the image name) can be imported.
- Import From	Here you can import images according to a **File List** or a **Multi page image**. Click on the folder button to the right of the display field. The names of the images are displayed in the **File Name** list below the display field.
Specifiy the Identifiers	Here you can enter and check all the settings you need to identify your images.

9.12.14.5 Import TXM

This function imports a txm file to ZEN.

Parameter	Description
Input	Selects a txm file for import.

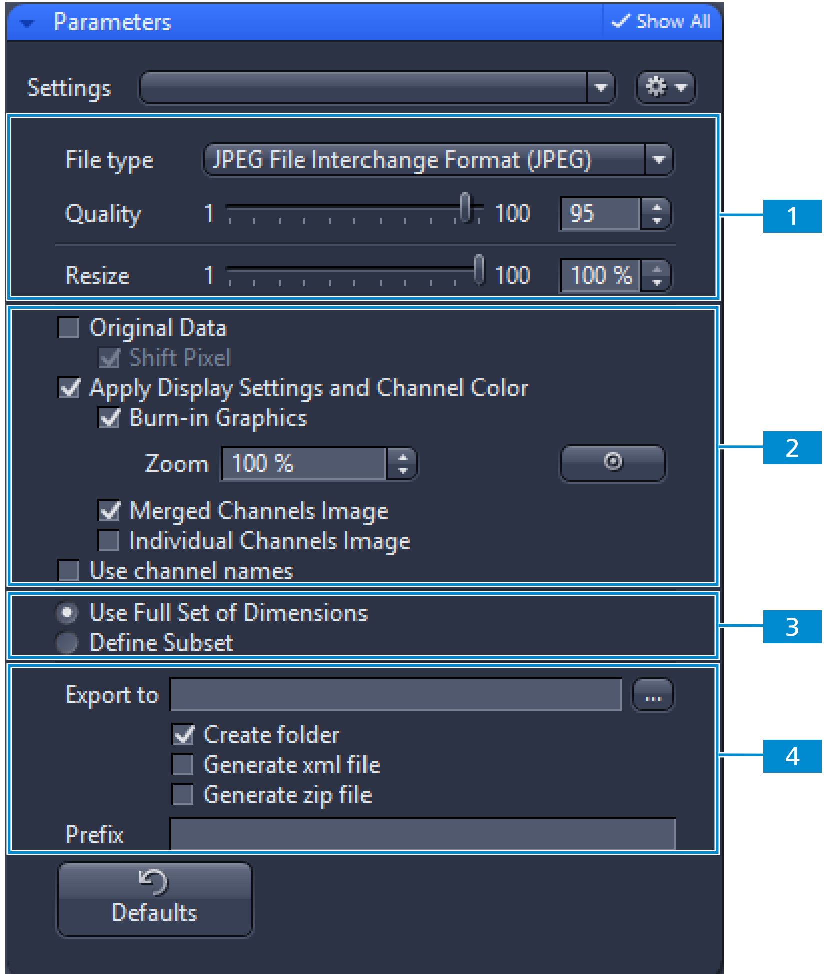
9.12.14.6 Convert TXM Files to CZI

This function converts txm images to czi.

Parameter	Description
Directory	Selects the folder with txm images that should be converted to czi. After the conversion, the images will again be stored in this folder.
Recursive	Converts also files in subfolders of the selected folder.
Overwrite	Overwrites already converted files.
Preview Only	Displays a preview of the images in the folder, but does not convert them.

9.12.14.7 Image Export

Using this method you can export single images into various file types so that you can continue to use them in other programs. Multidimensional images (multichannel, Z-stack, time lapse, tile images) are exported as individual images.



1 File Type Section
Here you define the settings for the file type, e.g. format or compression.
For more information, see *File Type section [▸ 208]*.
Depending on the file type you selected for the image export, the settings of the other parameters change.

2 Image Data Section
Here you define, how to deal with the image data during the export, e.g. whether you want to burn graphics firmly in the picture. For more information, see *Image Data section [▸ 209]*.

3 Dimensions Section
Here you specify, how to proceed with the dimensions, e.g. whether you want to export all the dimensions of an image, or only certain. For more information, see *Dimensions section [▸ 210]*.

4 Export to Section
Here you define other export settings, e.g. the export folder. For more information, see *Export to section [▸ 212]*.

9.12.14.7.1 File Type section

Name	Function
File Type	Select the desired file type from the dropdown list: ▪ **JPEG** (JPEG File Interchange Format) ▪ **BMP** (Windows Bitmap) ▪ **TIFF** (Tagged Image File Format) ▪ **Big TIFF** Tiff Format (64 bit) ▪ **PNG** (Portable Network Graphics) ▪ **WDP** (JPEG XR) ▪ **SUR** (DigitalSurf SUR) Note that various options are available depending on the file type you have selected.
Quality	Only available for the file types **JPEG** and **JPEG XR**. Enter the image quality using the slider or input field to influence the size of the file. Although low values result in very small files, image quality may be considerably reduced.
Resize	Adjust the image size in percent using the slider or input field.
Convert to 8 Bit	Only available for the file types **TIFF**, **BigTIFF**, **PNG** and **JPEG XR**. **Activated:** Converts a 16 bit gray level image into an 8 bit gray level image, or a 48 bit color image into a 24 bit color image.
Compression	Only available for the file type **TIFF** and **BigTIFF**. Select the compression method for reducing the data volume here:
- None	Retains the data volume of the original image. No compression is performed.
- LZW	Only available for the file type **TIFF**. Performs lossless compression in accordance with the Lempel-Ziv-Welch algorithm (LZW).
- ZIP	Only available for the file type **TIFF.** Performs lossless compression in accordance with the ZIP method.
- Loss less	Only available for the file type **BigTIFF.** Performs lossless compression in accordance with the Lempel-Ziv-Welch algorithm (LZW).
- Lossy	Only available for the file type **BigTIFF.** Performs lossy compression in accordance with the JPEG XR (extended range) method.
BigTIFF	Only available for the file type **BigTIFF**. **Activated:** Generates a BigTIFF image. The maximum image size is larger than 4GByte. **Deactivated:** Generates a TIFF image with maximum size of 4GByte.
TIFF Tiles	Only available for the file type **BigTIFF**. **Activated:** Generates new rectangle tiles for internal data handling. **Deactivated:** Combines tiles as stripes for internal data handling.

Name	Function
Pyramid	Only available for the file type **BigTIFF**. **Activated:** Calculates an image pyramid.
Merge All Scenes	Only available for the file type **BigTIFF**. **Activated:** Generates one image including all scenes. Single scene images will be generated, if the checkbox is deactivated.

9.12.14.7.2 Image Data section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Original Data	**Activated:** Exports the image with the original channel colors and the original display characteristic curve.
Shift Pixel	**Activated:** The pixel are shifted to 16 bit before converting to 8 bit. For example, a 14 bit image is first transformed to a 16 bit image which is then converted to a 8 bit image. The 14 bit range is mapped to the wohle 8 bit range. **Deactivated:** No shift takes place. A 14 bit image is treated as a 16 bit image and therefore the transformation to 8 bit covers only a reduced range.
Apply Display Curve and Channel Color	**Activated:** Exports the image with the changed channel color and display characteristic curve settings. These settings are applied to the pixel values of the exported images. They are particularly important if you want to use dark images with a dynamic range of more than 8 bits in other programs.

Parameter	Description
Apply Display Settings and Channel Color	
– **Burn-in Graphics**	**Activated:** Burns the graphic elements into the image. The pixels under the graphic element (e.g. scale bars) are overwritten. The burnt-in graphic elements cannot be subsequently modified.
– **Zoom**	Only available if **Burn-in Graphics** is activated. Sets the zoom of graphic elements for export. The default is 100%, which means that the graphical elements are rendered as they would if the viewer zoom was 100%. When the zoom increases, the graphical elements are rendered larger (e.g. with zoom 200, graphical elements are rendered twice as large, but it corresponds to the size they would have if the viewer zoom was 50%).
– **Get Zoom from Viewer**	Only available if **Burn-in Graphics** is activated. Gets the current zoom factor of the graphical elements in the 2D view.
– **Merged Channels Image**	Only visible for multichannel images. **Activated:** Exports the pseudo color image of all selected channels.

Parameter	Description
– **Individual Channels Image**	Only visible for multichannel images. **Activated**: Exports the individual colored image of all selected channels.
Use channel names	Only visible for multi-channel images. **Activated:** Integrates the channel name in the name of the exported image.
Use Full Set of Dimensions	**Activated:** Includes all dimensions of the original file.
Define Subset	**Activated:** Creates a subset of the data, depends on the dimensions available in the image, e.g. Channels, Region, Tiles.

At least one of the three checkboxes must be activated. If the **Merged Channels Image** and **Individual Channel Image** checkboxes are activated, you can export the individual colored images and the pseudo color images in a single step.

Parameter	Description
Export to	Sets path to the folder where images will be exported.
Create Folder	Creates a folder for your exported data.
Generate xml file	Additionally, creates an xml file containing the meta data of the image.
Generate zip file	Creates a .zip file to be exported.
Prefix	Adds the specified name as a prefix to the exported data. By default this is the name of the original image data.
Defaults	Sets the settings back to default.

9.12.14.7.3 Dimensions section

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Use Full Set of Dimensions	Select this option if you want to export all dimensions without changing them.
Define Subset	Select this option if you only want to export individual dimensions or subsets of individual dimensions.

Info

Each of the sections described below is only visible if the corresponding dimension is present in the input image.

Parameter	Description
Channels	Here you can select which channels of the input image you want to be used. All channels are selected by default. To deselect a channel, click on the relevant channel button.
Z-Position, **Time**, **Block**, **Scene**	Here you can select which parts of the input image you want to use for the resulting image.
- Extract All	If selected, all parts of the corresponding image are extracted.
- Extract Single	If selected, you can select a single image to be extracted.
- Extract Range	If selected, you can select a certain range of images to be extracted.
- Extract Multiple	If selected, you can select several continuous ranges and individual sections. Enter one or more sections that you want to select in the input field. To do this, enter the first section, followed by a minus sign, and then the last section. If you want to define an interval, after the last section enter a colon and then the interval. The entry "2-10:2" means that every second section is selected from section 2 to section 10. Enter a comma after the first section if you want to define another section. You can also select individual sections separated by commas. By entering "2-10:2,14-18,20,23", you select every second section from section 2 to section 10, followed by sections 14 to 18, as well as sections 20 and 23.
- Get current position	Adopts the position from the current display in the image area.
- Interval	**Activated:** Interval mode is active. The Interval spin box/input field appears. Enter the desired interval here. E.g. if you enter the value 2 only every 2nd value from the range is considered.
Region	Here you can select if you want to use the entire image or just a region (ROI) of the input image.
- Full	If selected this option, the full image is used for the new image.
- Rectangle region (ROI)	If selected this option, you can draw in a rectangle region of interest which will be used for creating a new image. If a rectangle region was drawn in you can see and change its coordinates by editing the **X/Y/W/H** input fields.
- Keep tiles	Has only an effect, if a region (ROI) is defined. **Activated:** Extracts the drawn in region including the complete tiles. This setting is recommend when you want to apply DCV processing functions on the resulting image.
Tiles	
- Export selected tiles	Exports the tiles according to the selected original image.
- Crop to selection and generate new tiles	Creates new tiles according to your entries in the fields **Colums/Rows** and **Overlap**.

Parameter	Description
- Colums /Rows	Only active if **Re-Tile** is activated. Defines, with how many rows and colums the re-tiling is performed.
- Overlap	Only active if **Re-Tile** is activated. Defines the overlap of the re-tiled tiles in %.

9.12.14.7.4 Export to section

Parameter	Description
Export to	The path of the export folder is displayed automatically in the display field. To change the file path, click on the ... button to the right of the display field.

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Create folder	**Activated:** Creates a separate folder with the name of the input image.
Create xml file	**Activated:** Creates two XML files containing the meta information relating to the input image. ▪ **Meta.xml** contains all additional information relating to the input image (e.g. hardware settings, dimensions). ▪ **Info.xml** only contains additional information relating to the exported individual images (e.g. names, dimensions, sizes).
Generate zip file	**Activated**: Creates a ZIP file in which all exported individual images, including the XML files, are saved.

Parameter	Description
Prefix	Here you can edit the prefix specified or enter a new name. The name of the original image is specified by default.

9.12.15 Image Analysis

The group **Image Analysis** provides different options to analyze images in single or batch mode. The measurement data can be embedded in the image, saved in a ***csv**-datalist, or saved as label image.

9.12.15.1 Analyze

This function is applicable only for one image: This must be selected in the **Input** tool. The measurement data are embedded in the image.

9.12.15.2 Analyze to File

This function is applicable only for one image: This must be selected in the **Input** tool. The measurement data is stored in a ***csv** list and is not embedded into the image.

The following file types are supported:

- CZI
- ZVI
- BMP
- TIF
- JPG

Parameter	Description
Data Folder	Folder where the ***csv**-data lists will be stored.
Base Name	Name of the data list.

9.12.15.3 Analyze Batch

This function allows the analysis of all images in a folder.

The function **Analyze Batch** can only be applied to images with the file type ***czi**.

The measured data are embedded in each original image.

Parameter	Description
Image Folder	Folder of the ***czi** images to be measured.

9.12.15.4 Analyze Batch to File

This function allows an analysis of all images in a folder. The measured data are stored in a ***csv** list and not embedded into the image.

The following file types are supported:

- CZI
- ZVI
- BMP
- TIF
- JPG

Parameter	Description
Image Folder	Folder of the images to be measured.
Data Folder	Folder where the ***csv**-data lists will be stored.
Base Name	Name of the data list.
Append Data	**Activated**: For each class, an accumulated csv-data list is stored with the measurement data of all images. **Deactivated**: For each image, one data list is stored per class.

9.12.15.5 Analyze to Label Image

The image processing functions **Analyze to Label Image** labels images based on an existing image analysis setting and the parameters which you select in the **Parameters** section.

Parameter	Description
Setting	Displays image analysis settings in the drop down list.
Initialization	Initializes the output image.
– One channel	Creates a one channel output image.
– One channel and copy first	Creates a one channel output image and copies the content of the first channel.
– One channel per class	Creates an output image with one channel per region class.
– One channel per class and copy	Creates an output image with one channel per region class and copies the content of the original channels of each class.
– All referenced channels	Creates all channels referenced in the image analysis setting.
– All referenced channels and copy	Creates all channels referenced in the image analysis setting and copies the content of the original image.
Label Mode	Selects the mode of labels applied to the image.
– Region - Pixel Type Maximum	Labels the region with the maximum value of the pixel type.
– Region - Label Value	Labels the region with the value specified by the **Label Value** slider/ input field.
– Region - Region ID	Labels the region with the region ID.
– Region - Region ID Color	Labels the region with the region ID color.
– Region - Region Class ID	Labels the region with the region class ID.
– Region - Region Class Color	Labels the region with the region class color.
– Contour - Pixel Type Maximum	Draws a contour with the maximum value of the pixel type.
– Contour - Label Value	Draws a contour with the value specified by the **Label Value** slider/ input field.
– Contour - Region ID	Draws a contour with the color defined by the region ID.
– Contour - Region ID Color	Draws a contour with the color defined by the region ID color.

Parameter	Description
– Contour - Region Class ID	Draws a contour with the color defined by the region class ID.
– Contour - Region Class Color	Draws a contour with the color defined by the region class color.
– Contour2 - Pixel Type Maximum	Draws a double contour with the value defined by the maximum value of the pixel type.
– Contour2 - Label Value	Draws a double contour with the value specified by the **Label Value** slider/ input field.
– Contour2 - Region ID	Draws a double contour with the color defined by the region ID.
– Contour2 - Region ID Color	Draws a double contour with the color defined by the region ID color.
– Contour2 - Region Class ID	Draws a double contour with the color defined by the region class ID.
– Contour2 - Region Class Color	Draws a double contour with the color defined by the region class color.
Pixel Type	Selects the pixel type for the output image.
– 8 Bit B/W	Creates a 8 bit black and white output image.
– 16 Bit B/W	Creates a 16 bit black and white output image.
– 24 Bit RGB	Creates a 24 bit colored output image.
– 48 Bit RGB	Creates a 48 bit colored output image.
– 32 Bit B/W Float	Creates a 32 bit black and white image.
Label Value	Selects the label value.

See also

Using Analyze to Label Image [▸ 108]

9.12.15.6 Analyze to Label Image Batch

The image processing functions **Analyze to Image Batch** labels images based on an existing image analysis setting and the parameters which you select in the **Parameters** section.

Parameter	Description
Setting	Displays image analysis settings in the drop down list.
Image Folder	Selects the folder of the images.
Initialization	Initializes the output image.
– One channel	Creates a one channel output image.
– One channel and copy first	Creates a one channel output image and copies the content of the first channel.

Parameter	Description
– One channel per class	Creates an output image with one channel per region class.
– One channel per class and copy	Creates an output image with one channel per region class and copies the content of the original channels of each class.
– All referenced channels	Creates all channels referenced in the image analysis setting.
– All referenced channels and copy	Creates all channels referenced in the image analysis setting and copies the content of the original image.
Label Mode	Selects the mode of labels applied to the image.
– Region - Pixel Type Maximum	Labels the region with the maximum value of the pixel type.
– Region - Label Value	Labels the region with the value specified by the **Label Value** slider/ input field.
– Region - Region ID	Labels the region with the region ID.
– Region - Region ID Color	Labels the region with the region ID color.
– Region - Region Class ID	Labels the region with the region class ID.
– Region - Region Class Color	Labels the region with the region class color.
– Contour - Pixel Type Maximum	Draws a contour with the maximum value of the pixel type.
– Contour - Label Value	Draws a contour with the value specified by the **Label Value** slider/ input field.
– Contour - Region ID	Draws a contour with the color defined by the region ID.
– Contour - Region ID Color	Draws a contour with the color defined by the region ID color.
– Contour - Region Class ID	Draws a contour with the color defined by the region class ID.
– Contour - Region Class Color	Draws a contour with the color defined by the region class color.
– Contour2 - Pixel Type Maximum	Draws a double contour with the value defined by the maximum value of the pixel type.
– Contour2 - Label Value	Draws a double contour with the value specified by the **Label Value** slider/ input field.
– Contour2 - Region ID	Draws a double contour with the color defined by the region ID.

Parameter	Description
– Contour2 - Region ID Color	Draws a double contour with the color defined by the region ID color.
– Contour2 - Region Class ID	Draws a double contour with the color defined by the region class ID.
– Contour2 - Region Class Color	Draws a double contour with the color defined by the region class color.
Pixel Type	Selects the pixel type for the output image.
– 8 Bit B/W	Creates a 8 bit black and white output image.
– 16 Bit B/W	Creates a 16 bit black and white output image.
– 24 Bit RGB	Creates a 24 bit colored output image.
– 48 Bit RGB	Creates a 48 bit colored output image.
– 32 Bit B/W Float	Creates a 32 bit black and white image.
Label Value	Selects the label value.
Format	Selects the format of the output image. You can save your image(s) as: ▪ czi ▪ png ▪ czi and png

See also

Using Analyze to Label Image [▸ 108]

9.12.15.7 Analyze Interactive Batch

This function allows the interactive analysis of all images in a folder. If steps of the image analysis are marked as interactive in the image analysis setting, this function opens the *Image Analysis Wizard [▸ 777]* with these steps. You can then adjust your analysis for each of the images. If you deselect one of the interactive steps during the process, this step will not be shown for the analysis of the following images. The image analysis setting remains unchanged by the changes made during **Analyze Interactive Batch**.

The measured data are embedded in each original image.

Parameter	Description
Image Folder	Folder of the *__czi__ images to be measured.

9.12.16 Lightsheet

This group of processing functions allows you to analyze multiview dual side illuminated Lightsheet images. The following functions are available:

- *Dual Side Fusion [▶ 218]*
- *Lightsheet Convert Dimensions [▶ 219]*
- *Lightsheet Processing [▶ 220]*
- *Maximum Intensity Projection [▶ 228]*

9.12.16.1 Dual Side Fusion

This function merges the image done with the lightsheet from the right side with the image of the left side. It can be used for the following datasets acquired with Dual Side illumination:

- Image (Snap function)
- Time Series
- Z-Stacks
- Combinations of time series and z-stacks
- Multiview experiments in combination with time series

Parameter	Description
Settings	Allows handling predefined settings (templates). See also *General Settings [▶ 90]*.
– New	Creates a new setting. Enter a name for the setting.
– New from Template	Creates a new setting based on an existing template.
– Rename	Renames the setting.
– Save	Saves a modified setting under the current name. An asterisk indicates the modified state.
– Save As	Saves the current setting under a new name.
– Import	Imports an existing setting.
– Export	Exports the current setting.
– Delete	Deletes the current setting.
Mean	The average intensity for each pixel is calculated using the values of both illumination sides of the fusion.
DFT	Discrete Fourier Transform fusion according to publication. Fine structures and details are more visible after the fusion, which might be worsened when using Mean Fusion. However, this process can create noticeable artifacts in the resulting image.
Maximum	The intensity of each pixel of both illumination images is compared and the value with the higher intensity is used for the resulting image of the fusion.

Parameter	Description
Fusion Subset in X	Only available for **Mean** or **Maximum** fusion. Selects which area of the left and the right illumination image should be used for fusion.
– Current View	Displays the selected view of a Multiview image in the **2D** tab which is used for fusion subset in x.. **Note:** The position of the sliders **Left** and **Right** as well as the **Blending** value have to be defined for each view individually.
– Left	Selects the area of the left illumination side which is used for fusion. The spin box shows the cut-off x pixel value. The cutoff is shown in the **2D** vie with a red line. **Note:** It is best to set the illumination slider of the **Dimensions** view control to **1** to see the left-side illuminated image.
– Right	Selects the area of the right illumination side which is used for fusion. The spin box shows the cut-off pixel value of the y axis. The cutoff is shown in the **2D** view with a blue line. **Note:** It is best to set the illumination slider of the **Dimensions** view control to **2** to see the right-side illuminated image.
– Blending	Defines the number of pixels used within an existing overlap between left and right illumination to crossfade both illuminations sides. **Note:** To avoid a visible change in intensity along the two cut off lines for **Mean Fusion** with **Fusion Subset in X**, an overlap of both illumination sides should remain in the center of the image.

9.12.16.2 Lightsheet Convert Dimensions

This function allows you to convert some dimensions into channels.
The following dimensions are available:

- View (from Multiview)
- Illumination (from Dual side)

The following dimensions are unavailable:

- Time (from Time series)
- Z (from Z-Stack)
- Tile (from Tiling)
- Position (from Positions)

Parameter	Description
Settings	Allows handling predefined settings (templates). See also *General Settings [▸ 90]*.
– New	Creates a new setting. Enter a name for the setting.
– New from Template	Creates a new setting based on an existing template.
– Rename	Renames the setting.
– Save	Saves a modified setting under the current name. An asterisk indicates the modified state.

Parameter	Description
– Save As	Saves the current setting under a new name.
– Import	Imports an existing setting.
– Export	Exports the current setting.
– Delete	Deletes the current setting.
View to channel	Converts views from a **Multiview** data set into channels.
Illumination to channel	Converts illuminations from a **Dual Side** data set into channels.

9.12.16.3 Lightsheet Processing

Lightsheet processing allows you to deconvolve, register, and fuse Multiview images.

Parameter	Description
Settings	Allows handling predefined settings (templates). See also *General Settings [▸ 90]*.
– New	Creates a new setting. Enter a name for the setting.
– New from Template	Creates a new setting based on an existing template.
– Rename	Renames the setting.
– Save	Saves a modified setting under the current name. An asterisk indicates the modified state.
– Save As	Saves the current setting under a new name.
– Import	Imports an existing setting.
– Export	Exports the current setting.
– Delete	Deletes the current setting.
Deconvolution	**Activated:** Allows to deconvolve Lightsheet data (single views) of the following formats: ▪ Multiview experiments before registration and fusion (single or dual side illumination): Deconvolution is performed for each frame from all z-stacks and all views. The process uses a point spread function (PSF) valid for single view images. ▪ Multiview experiments after registration and fusion: Deconvolution is performed on the fused dataset, hence only one z-stack needs to be processed. It uses a star-like point spread function (PSF) computed from the fused Multiview image. For more information, see *Deconvolution (adjustable) [▸ 124]*.
Multiview	**Activated:** Allows registration, fusion, and deconvolution of Multiview images.

Parameter	Description
– Registration options	Allows to select the alignment method: ▪ *Landmark based alignment [▸ 221]* ▪ *Intensity based aligment [▸ 223]* ▪ *Use Registration from File [▸ 225]* ▪ *Interactive Registration [▸ 226]* The following datasets can be processed with this function: ▪ Multiview datasets, single side illumination ▪ Multiview datasets, dual side illumination, Dual Side without Fusion ▪ Multiview datasets, dual side illumination, Dual Side with Fusion ▪ Multiview datasets with Deconvolution ▪ Multiview datasets without Deconvolution The **Registration** generates the transformation matrices so that all views are matched to overlay. As a result, a dataset is provided in which all z-stacks of all views should already largely overlay. The reference at which registrations starts is always View 1 of the dataset. This image can be used for control. If one or more views do not have the same alignment, registration was not successful and the parameters need to be adjusted. The **Registration** must always be done before the **Fusion** of the data.

9.12.16.3.1 Landmark based alignment

Registration is done based on structural information within the image content.

Parameter	Description
Parameter Settings	Setting of alignment parameters.
– Registration Channel	Selects the registration channel. Use a channel with fiducials to which other channels will be registered to. **Note:** A selection of more than one channel for the registration process is possible. For each selected channel an individual registration will be performed and, therefore, it is advisable to use one channel for the final registration. To test which channel will lead to the best results, all channels can be selected for later comparison of the results.
– Threshold	Selects thresholds for the brightness of fiducials (beads).
– Size	Selects thresholds for the size of fiducials (beads).
– Size Variations	Selects thresholds for the variation in size difference.
– Dual side fusion	This operation merges the image done with the lightsheet from the right side with the image of the left side. See also *Dual Side Fusion [▸ 218]*.
– Fusion Subset in X	**Activated:**Selects which area of the left and the right illumination image should be used for fusion. Only available for **Mean** or **Maximum** fusion. See also the description in *Dual Side Fusion [▸ 218]*.

Parameter	Description
– Ignore Sample	**Activated:** Uses a function to find incorrectly labeled fiducials and eliminate them for registration.
– Expand to maximum value	**Activated:** The volume of the resulting dataset after registration and fusion is determined and additional black pixels (x,y) and z-slices are added to the views during registration. This prevents loss of data for later fusion.
– Scaling X=Y=Z	**Activated:** The dimensions are downsized to the smallest to have the same size. If the scaling in Deconvolution or PSF settings is changed, the latter overrides the scaling settings here.
– Individual Registration for every Time Point	**Activated:** Each time point generates a transformation matrix and each time point is registered individually. The time necessary for registration is multiplied by the number of time points and can increase substantially. However, if the motorized movement of the sample by the system has changed (e.g. due to thermic effects on the system: not fully warmed up at start, incubation, air conditioning of the room), the **Individual Registration For Every Time Point** option is necessary. **Deactivated:** One transformation matrix is generated for all the following time points based on the first time point. As a result, processing for registration has only to be done once which reduces processing time.

Parameter	Description
Input settings	A subset of a present time series can be selected for the Registration and Fusion processing.
– Processing Subset Time	Truncates time points.
– Processing Subset Steps	Defines time-to-time point step size.
– Scaling correction (µm)	Sets the geometric scaling between X/Y and Z.
Fusion	Fuses registered views in one z-stack or one z-stack over time.
– None	No fusion is performed after Registration and Dual Side Fusion.
– Mean Fusion	The pixel in the fused image is determined by averaging the intensity level of the pixels for the involved views.
– Mean Fusion + Multiview DCV	Only available if the dataset has not undergone deconvolution before. The pixel in the fused image is determined by averaging the intensity level of the pixels of the involved views. After fusion, a deconvolution is performed. The *Deconvolution [▸ 124]* interface is displayed if selected.
– Blending	Controls the amount of Blending (smoothing) by fading the borders of all z-stacks in order to prevent visible edge effects. **Note:** The value for Blending should be as low as possible with typical values from 20 to 80. This needs to be tested for every dataset.

Parameter	Description
– Fusion Subset in Z	**Activated:** Defines which portion of the z-stacks of the contributing views of the experiment are used for the fusion process.
– Current View	Selects the current view. For each view the z-range can be set via slider, spin boxes, or mouse click in the image. Registration will not be affected by the z-range settings. Note that for later deconvolution the calculated point spread function (PSF) of a Multiview experiment assumes that all views equally contribute to all areas of the image. This is not the case when using Subset Z and might result in artefacts of the deconvolution result.
– Save unfused data	**Activated:** Saves the registered image in addition to the fused image.

See also

Deconvolution (adjustable) [▸ 124]

9.12.16.3.2 Intensity based aligment

Parameter	Description
Parameter Settings	Setting of alignment parameters.
– Registration Channel	Selects the registration channel. Use a channel with fiducials to which other channels will be registered to. **Note:** A selection of more than one channel for the registration process is possible. For each selected channel an individual registration will be performed and, therefore, it is advisable to use one channel for the final registration. To test which channel will lead to the best results, all channels can be selected for later comparison of the results.
– Preregistration	Preregistration performs the process in a 2D maximum intensity projection of the z-stacks. It is advisable to first perform a **Pre-Registration** to move similar structures, which might facilitate the **Registration**, already into close proximity.
– None	No Preregistration is performed.
– Hardware Rotation	The views are rotated solely based on the information from the rotation positioning of the sample holder which is saved in the metadata.
– Trans Hardware Rotation	The views are rotated solely based on the information from the rotation positioning of the sample holder which is saved in the metadata. Additionally, the x, y, and z axis are moved to match the views.
– Correlation Hardware Rotation	The views are rotated solely based on the information from the rotation positioning of the sample holder which is saved in the metadata. Additionally, the x, y, and z axis are moved to match the views. Cross-correlation is used to determine the translation in x, y, and z.
– Registration Method	Registration uses the complete 3D information of the z-stacks.
– Use Pre Register Only	No Registration process is performed and only the result of the Pre-Registration is used.

Parameter	Description
– Translation	The registration only corrects along the x, y, and z axis.
– Trans Rotation	The registration corrects along the x, y, and z axis and also the rotation along x and y.
– Dual side fusion	Merges the image done with the lightsheet from the right side with the image of the left side. See also *Dual Side Fusion [▸ 218]*.
– Fusion Subset in X	**Activated:** Selects which area of the left and the right illumination image should be used for fusion. Only available for **Mean** or **Maximum** fusion. See also the description in *Dual Side Fusion [▸ 218]*.
– Ignore Sample	**Activated:** Uses a function to find incorrectly labeled fiducials and eliminate them for registration.
– Expand to maximum value	**Activated:** The volume of the resulting dataset after registration and fusion is determined and additional black pixels (x,y) and z-slices are added to the views during registration. This prevents loss of data for later fusion.
– Scaling X=Y=Z	**Activated:** The dimensions are downsized to the smallest to have the same size. If the scaling in Deconvolution or PSF settings is changed, the latter overrides the scaling settings here.
– Individual Registration for every Time Point	**Activated:** Each time point generates a transformation matrix and each time point is registered individually. The time necessary for registration is multiplied by the number of time points and can increase substantially. However, if the motorized movement of the sample by the system has changed (e.g. due to thermic effects on the system: not fully warmed up at start, incubation, air conditioning of the room), the **Individual Registration For Every Time Point** option is necessary. **Deactivated:** One transformation matrix is generated for all the following time points based on the first time point. As a result, processing for registration has only to be done once which reduces processing time.

Parameter	Description
Input settings	A subset of a present time series can be selected for the Registration and Fusion processing.
– Processing Subset Time	Truncates time points.
– Processing Subset Steps	Defines time-to-time point step size.
– Scaling correction (µm)	Sets the geometric scaling between X/Y and Z.
Fusion	Fuses registered views in one z-stack or one z-stack over time.
– None	No fusion is performed after Registration and Dual Side Fusion.
– Mean Fusion	The pixel in the fused image is determined by averaging the intensity level of the pixels for the involved views.

Parameter	Description
– Mean Fusion + Multiview DCV	Only available if the dataset has not undergone deconvolution before. The pixel in the fused image is determined by averaging the intensity level of the pixels of the involved views. After fusion, a deconvolution is performed. The *Deconvolution [▸ 124]* interface is displayed if selected.
– Blending	Controls the amount of Blending (smoothing) by fading the borders of all z-stacks in order to prevent visible edge effects. **Note:** The value for Blending should be as low as possible with typical values from 20 to 80. This needs to be tested for every dataset.
– Fusion Subset in Z	**Activated:** Defines which portion of the z-stacks of the contributing views of the experiment are used for the fusion process.
– Current View	Selects the current view. For each view the z-range can be set via slider, spin boxes, or mouse click in the image. Registration will not be affected by the z-range settings. Note that for later deconvolution the calculated point spread function (PSF) of a Multiview experiment assumes that all views equally contribute to all areas of the image. This is not the case when using Subset Z and might result in artefacts of the deconvolution result.
– Save unfused data	**Activated:** Saves the registered image in addition to the fused image.

9.12.16.3.3 Use Registration from File

When the registration for the dataset is already determined and the transformation matrix is saved as an .xml file (Filename.czi.xml), this option can be selected.

Parameter	Description
Parameter Settings	Setting of alignment parameters.
– Select a registration file […]	Opens the Windows File Explorer to select a file.
– Dual side fusion	Merges the image done with the lightsheet from the right side with the left side image. See also *Dual Side Fusion [▸ 218]*.
– Fusion Subset in X	**Activated:**Selects which area of the left and the right illumination image should be used for fusion. Only available for **Mean** or **Maximum** fusion. See also the description in *Dual Side Fusion [▸ 218]*.
– Expand to maximum value	**Activated:** The volume of the resulting dataset after registration and fusion is determined and additional black pixels (x,y) and z-slices are added to the views during registration. This prevents loss of data for later fusion.

Parameter	Description
Input settings	A subset of a present time series can be selected for the Registration and Fusion processing.
– Processing Subset Time	Truncates time points.

Parameter	Description
– Processing Subset Steps	Defines time-to-time point step size.
– Scaling correction (µm)	Sets the geometric scaling between X/Y and Z.
Fusion	Fuses registered views in one z-stack or one z-stack over time.
– None	No fusion is performed after Registration and Dual Side Fusion.
– Mean Fusion	The pixel in the fused image is determined by averaging the intensity level of the pixels for the involved views.
– Mean Fusion + Multiview DCV	Only available if the dataset has not undergone deconvolution before. The pixel in the fused image is determined by averaging the intensity level of the pixels of the involved views. After fusion, a deconvolution is performed. The *Deconvolution [▸ 124]* interface is displayed if selected.
– Blending	Controls the amount of Blending (smoothing) by fading the borders of all z-stacks in order to prevent visible edge effects. **Note:** The value for Blending should be as low as possible with typical values from 20 to 80. This needs to be tested for every dataset.
– Fusion Subset in Z	**Activated:** Defines which portion of the z-stacks of the contributing views of the experiment are used for the fusion process.
– Current View	Selects the current view. For each view the z-range can be set via slider, spin boxes, or mouse click in the image. Registration will not be affected by the z-range settings. Note that for later deconvolution the calculated point spread function (PSF) of a Multiview experiment assumes that all views equally contribute to all areas of the image. This is not the case when using Subset Z and might result in artefacts of the deconvolution result.
– Save unfused data	**Activated:** Saves the registered image in addition to the fused image.

9.12.16.3.4 Interactive Registration

The recorded views of an experiment are registered manually. The views of one channel at one time point of the experiment are the basis for the procedure. The z-stacks of the individual views are rotated based on the rotation information of the files metadata and transformed into channels, resulting in one maximum intensity projection image. Within this newly generated image, the views, now behaving as channels, can be moved in x, y, z and rotated to overlay each other. When all views are laid on top of each other, the **Apply** button uses the adjusted parameters to register, and when required to fuse, the views for all time points of the experiment. An .xml file is created which can be used for the **Use registration from file** option. This .xml file can be found along with the processed data in the result folder.

Parameter	Description
Parameter Settings	Setting of alignment parameters.

Parameter	Description
– Registration Channel	Selects the registration channel that should be used to manually overlay all views of the image. **Note:** Only one channel can be used for this procedure, all other channels will not be displayed.
– Current Time Point	Indicates the selected time point of a time series as set with the time slider in the **Dimensions** tab.
– Front View	Converts views into channels and generates a maximum intensity projection along the z-axis of channels displaying the x- (horizontal) and y- (vertical) axis of channels representing. **Note:** It is advisable to assign different colors to the channel to ease up alignment process. Channels can be turned on/off in the **Dimensions** tab.
– Side View	Converts views into channels and generates a maximum intensity projection along the x-axis displaying the z- (horizontal) and y- (vertical) axis of channels representing. **Note:** It is advisable to assign different colors to the channel to ease up alignment process. Channels can be turned on/off in the **Dimensions** tab.
– View #	Selects the view to be aligned to View 1. View 1 is the master view and the reference channel and cannot be selected or moved. View # can be moved by translation and rotation against View 1. It does not matter if alignment is started with **Front View** or **Side View**. Three iterations between **Front View** and **Side View** alignment are recommended.
– Alignment X	Shifts the selected view against View 1 in X. Not available for **Side View**.
– Alignment Y	Shifts the selected view against View 1 in Y.
– Alignment Z	Shifts the selected view against View 1 in Z. Not available for **Front View**.
– Rotation	Rotates the selected view around the position of the red cross overlay within the image of the **2D** tab.
– Reset	Moves all views back to the position where the maximum intensity projection was generated.
– Preview	Produces a new image of the channel and the time point which is registered with the provided settings. The image will contain fused views when **Mean Fusion** is selected for **Fusion**.
– Dual side fusion	Merges the image done with the lightsheet from the right side with the image of the left side. See also *Dual Side Fusion [▸ 218]*.
– Fusion Subset in X	**Activated:** Selects which area of the left and the right illumination image is used for fusion. Only available for **Mean** or **Maximum** fusion. See also the description in *Dual Side Fusion [▸ 218]*.
– Expand to maximum value	**Activated:** The volume of the resulting dataset after registration and fusion is determined and additional black pixels (x,y) and z-slices are added to the views during registration. This prevents loss of data for later fusion.

Parameter	Description
– Scaling X=Y=Z	**Activated:** The dimensions are downsized to the smallest to have the same size. If the scaling in Deconvolution, PSF settings is changed, the latter will override the scaling settings here.

Parameter	Description
Input settings	A subset of a present time series can be selected for the Registration and Fusion processing.
– Processing Subset Time	Truncates time points.
– Processing Subset Steps	Defines time-to-time point step size.
– Scaling correction (µm)	Sets the geometric scaling between X/Y and Z.
Fusion	Fuses registered views in one z-stack or one z-stack over time.
– None	No fusion is performed after Registration and Dual Side Fusion.
– Mean Fusion	The pixel in the fused image is determined by averaging the intensity level of the pixels for the involved views.
– Mean Fusion + Multiview DCV	Only available if the dataset has not undergone deconvolution before. The pixel in the fused image is determined by averaging the intensity level of the pixels of the involved views. After fusion, a deconvolution is performed. The *Deconvolution [▸ 124]* interface is displayed if selected.
– Blending	Controls the amount of Blending (smoothing) by fading the borders of all z-stacks in order to prevent visible edge effects. **Note:** The value for Blending should be as low as possible with typical values from 20 to 80. This needs to be tested for every dataset.
– Fusion Subset in Z	**Activated:** Defines which portion of the z-stacks of the contributing views of the experiment are used for the fusion process.
– Current View	Selects the current view. For each view the z-range can be set via slider, spin boxes, or mouse click in the image. Registration will not be affected by the z-range settings. Note that for later deconvolution the calculated point spread function (PSF) of a Multiview experiment assumes that all views equally contribute to all areas of the image. This is not the case when using Subset Z and might result in artefacts of the deconvolution result.
– Save unfused data	**Activated:** Saves the registered image in addition to the fused image.

9.12.16.4 Maximum Intensity Projection

The function allows you to perform a Maximum Intensity Projection (MIP) for selected dimensions.

Parameter	Description
Settings	Allows handling predefined settings (templates). See also *General Settings [▸ 90]*.

Parameter	Description
– New	Creates a new setting. Enter a name for the setting.
– New from Template	Creates a new setting based on an existing template.
– Rename	Renames the setting.
– Save	Saves a modified setting under the current name. An asterisk indicates the modified state.
– Save As	Saves the current setting under a new name.
– Import	Imports an existing setting.
– Export	Exports the current setting.
– Delete	Deletes the current setting.
Online Batch Processing	**Activated:** Starts the online batch processing.
Coordinate	Selects the coordinate for which the maximum intensity projection (MIP) is performed.
– Z	MIP of a z-stack.
– C	MIP of channels.
– T	MIP of a time series.
– I	MIP of Illumination sides.
– V	MIP of views.
Range	Sets the range of the dimension to be used for the maximum intensity projection. The range can be set with the two slider bars or the spin boxes.

9.12.17 Batch Processing Functions

9.12.17.1 Change scaling

This method is only available for batch processing.

Parameter	Description
X Scaling The following units are possible:	Changes the scaling of the image in X-direction.
- m/px	Changes the unit (length per pixel).
- cm/px	
- mm/px	
- µm/px	

Parameter	Description
- nm/px	
- pm/px	
- i/px	
- mil/px	
Y Scaling The following units are possible:	Changes the scaling of the image in Y-direction.
- m/px	Changes the unit (length per pixel).
- cm/px	
- mm/px	
- µm/px	
- nm/px	
- pm/px	
- i/px	
- mil/px	
Z Scaling The following units are possible:	Changes the scaling of the image in Z-direction.
- m/px	Changes the unit (length per pixel).
- cm/px	
- mm/px	
- µm/px	
- nm/px	
- pm/px	
- i/px	
- mil/px	
Pick scaling from file	Applies the scaling from another czi-file.

9.12.17.2 Image Analysis Program

This method is only available for batch processing.

Parameter	Description
Setting	Selects an existing image analysis setting.
Apply	Applies the selected setting to the highlighted dataset. The defined classes and class names are displayed.
Class-Tree	Displays the class-tree of the selected image analysis setting.

9.12.17.3 Analyze to File

Parameter	Description
Setting	Selects an existing image analysis setting.
Data Folder	Specifies the folder where the analyzed csv-files will be stored.
Base Name	Specifies the prefix of the file name for the analyzed csv-files.

9.12.17.4 Draw Scale Bar Annotation

This method is only available for batch processing.

Parameter	Description
Distance from document bounds	Sets the distance of the Scale Bar from the bounds of the image (in pixel).
Alignment	Sets the alignment of the Scale Bar.
- Top Left	
- Top Center	
- Top Right	
- Middle Left	
- Middle Center	
- Middle Right	
- Bottom Left	
- Bottom Center	
- Bottom Right	
Orientation	Sets the orientation of the Scale Bar.
- Horizontal	
- Vertical	

9.12.17.5 Split Multiblock Image (for images until ZEN 2.1)

Parameter	Description
Split Mode	Chooses the mode how to split the multiblock image (in pixel).
- Homogeneous groups	Splits the multiblock image into the single dimensions. The blocks will remain.
- Single blocks	Splits the multiblock image into single blocks.
Target Folder	Specifies the folder where the split images are stored. The path of the destination folder is displayed automatically in the display field. To change the folder, click on the button to the right of the display field.
Defaults	Restores the default values for the batch method.

9.12.17.6 Undo Stitching

This method is only available for batch processing.

Undoes a previously applied stitching of the image.

No method parameters necessary.

9.12.17.7 Attach PSF (Point Spread Function)

This method is only available for batch processing.

This method attaches a previously created PSF to the image file.

Parameter	Description
Channel	Selects the channel where the PSF should be attached.
PSF File	Selects the PSF-file created by the image processing function Create PSF.

10 Importing/ Exporting Images

10.1 Workflow Export/Import

This example describes the workflow for the **Image Export**. The typical workflow is the same for both export and import of images.

Prerequisite ✓ You have selected the **Processing** tab.

1. In the **Method** tool, open the **Export/Import** group and select the **Image Export** method.
2. Under **Method Parameters > Parameters**, set the desired export settings, e.g. file type, quality, export folder, etc..
3. Under **Image Parameters > Input**, select the image you want to export. To do this, click on the small preview image within the **Input** tool. You will see a preview of all the open images. To select an image, click on the image that you want to use. This is only necessary if you have several images open simultaneously. By default, the image currently selected is always used as the input image.
4. **Export the image**
   On the top part of the **Processing** tab, click on the **Apply** button.

You have successfully exported the selected image.

10.2 Exporting Images

Using the **Quick Export** function you can export images automatically with a single click of the mouse, without setting the method parameters.

Prerequisite ✓ You have acquired or opened an image.

1. In the **Right Tool Area** in the **Images and Documents** tool, click on the **Quick Export** button .
   Alternatively, you can click on the **Quick Export** entry via the **File > Export/Import**.

The selected image is automatically exported with the default settings of the **Image Export** method (JPEG, quality 95%, size 100%). The image can then be found in a subfolder within the Windows image folder (**…/User/My Pictures**).

All images have 300 dpi.

Info

If you export a time lapse image using the Quick Export function, a movie is automatically generated using the default values of the **Movie Export** method.

10.3 Exporting Movies

Using the **Quick Export** function you can export movies automatically with a single click of the mouse, without setting the method parameters.

Prerequisite ✓ You have acquired or opened an image from a Time Series or a Z-stack image.

1. In the **Right Tool Area** in the **Images and Documents** tool, click on the **Quick Export** button .
   Alternatively, you can click on the **Quick Export** entry on the **File > Export/Import**.

The selected experiment is automatically exported with the default settings of the **Movie Export** method (AVI, original size). The movie can then be found in a subfolder within the Windows image folder (**…/User/My Videos**).

See also

Movie Export [▹ 195]

10.4 Exporting Multichannel Images

Here you will find out how to export individual images from a multichannel image with three channels and save them automatically in a **ZIP** archive. You will also discover how to export the whole multichannel image (pseudo color image) as an individual image.

Prerequisite ✓ You have acquired or opened a multichannel image.

1. On the **Processing** tab, open the parameters for **Image Export** (*Ctrl+6* or via the **File > Export/Import > Export**).
   → You will see the default settings of the parameters for image export. Make sure that the **Show All** mode has been activated.
2. Activate the **Individual Channels Image** checkbox.
   → An image will be exported from each channel. The **Multichannel Image** checkbox is activated by default. This means that the pseudo color image (mixed color image from all channels) will also be exported as an individual image.
3. Activate the **Generate zip file** checkbox under **Export to**. Activated the **Create folder** checkbox, to create a subfolder with the name of the prefix.
   → The ZIP archive is saved in the subfolder.
4. Click on the **Apply** button at the top of the **Processing** tab.

You have exported the images of the individual channels and the pseudo color image of your multichannel image and automatically saved them in a ZIP archive. The ZIP archive containing the 4 images can be found in the export folder indicated.

10.5 Exporting Multiscene Images

You can export multiscene images into the following file formats:

- JPEG
- BMP
- TIFF
- Big TIFF
- PNG
- WDP
- SUR

Note that export of images to most of these formats is subject to limits of the file size that can be created (typically ca. 2 Gb). For this reason, we recommend to use the Big TIFF-format. If this is not possible because the Big TIFF-format does not fit your needs, check if the image size does not exceed the resources of your computer when exporting your preferred format. A message will inform you, when the size exceeds your resources. You have the following options to work around:

- With the **Resize** slider, resize the resolution to scale the image down.
- Activate **Original Data**, deactivate **Apply Display Settings and Channel Color**.
- With a rectangle, select a region of interest to minimize the image itself.

- Activate the **Re-Tile** radio button to cut the image again into tiles using the row and column options.

You can extract different scenes and regions of the image.

Prerequisite ✓ You have loaded a multiscene image.

1. On the **Processing** tab, open the parameters for the method **Export/Import | Image Export**. Alternatively, click *Ctrl+6* or via the **File** menu **| Export/Import | Export**.
   → You will see the default settings of the parameters for image export. Make sure that the **Show All** mode has been activated.

   → In the **Export** section, the path of the export folder is displayed automatically in the display field.
2. In the **File type** section, define the format for your image to be exported.
   → According to your selection, the functionalities might change.
3. If the format Big TIFF is selected, activate the **Merge all Scenes** checkbox to combine all scenes of your ROI in one image. Deactivate the checkbox to export single files per scene.
4. Activate the **Define Subset** radio button .
   → The **Scene**, the **Region** and the **Tiles** parameters are displayed.
5. In the **Scene** section, from the drop down menu, select which parts of the input image you want to use for the resulting image.
6. In the **Region** section, from the drop down menu, select if you want to export the full image or a rectangle region. If you select **Rectangle region**, with the left mouse button, you can drag a rectangle in the image according to your needs.
7. Click the **Apply** button.

You have exported one or more images to the export folder.

The naming follows a naming convention.

Naming convention of the exported file

The name of the exported file follows a convention: `<Prefix>_<Suffix>.<File Format>`. Example: TMA1_m1.tif

If there are no dimensions, the image name will not contain a suffix. Example: TMA1.tif

Image name of exported file	Description of the exported image
<Prefix>**_m<no.>**	Contains re-tile data. The count starts from top left to right and in the next row again from left to right.
<Prefix>**_c<no.>**	Contains channels.
<Prefix>**_t<no.>**	Contains time series.
<Prefix>**_z<no.>**	Contains z-stacks.
<Prefix>**_ORG_<no.>**	Contains original data.

The prefix is the image name you entered in the **Prefix field** in the **Tiles** section. Per default, the prefix field contains the image name. The suffix contains all image information. Example: If you export an z-stack with channel 1 and channel 2, and activate re-tile, the image name of one exported image could be: TMA1_z02c1+2m1.tif

See also

- Dimensions section [▸ 199]
- Export to section [▸ 212]
- File Type section [▸ 208]

10.6 Importing Z-Stack Images

Prerequisite ✓ You have saved the individual images of a Z-stack in a folder on your computer. The images have been named systematically, e.g. Image_Z0, Image_Z01, etc...

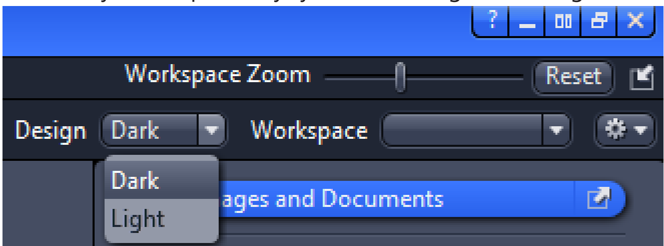
1. On the **Processing** tab, open the parameters for **Image Import** (or via the **File > Export/ Import > Import**).
   → You will see the default settings of the parameters for **Image Import**.
2. Activate the **Z-stack** checkbox. Deactivate all the other dimensions.
3. Enter the interval for the Z-stack. The number of planes is set automatically if the images have been named systematically.
4. In the **Import from** section, select the folder that contains the individual images of your Z-stack image.
   → The individual images are displayed automatically in the list under the import directory.
5. Click on the **Check Consistency** button. This allows you to check whether the images can be imported correctly.
   → A check mark appears after each file name in the list. You can import the individual images.
6. Click on the **Apply** button at the top of the **Processing** tab.

The individual images are imported and combined to form a Z-stack image. You have successfully imported a Z-stack image from individual images.

11 Customizing the Application

11.1 Selecting a Screen Design

In the upper right corner of the program window under **Design** you can select a **Light** or **Dark** screen layout . Optionally, you can change the design with the shortcut *Ctrl+D*.



11.2 Customizing Toolbar

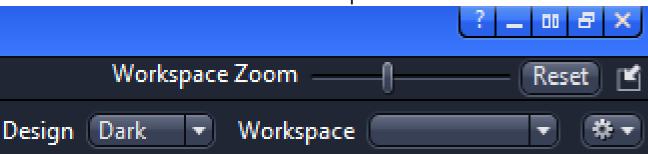
Prerequisite
- ✓ You are in the **Tools** menu | **Customize Application** dialog.
- ✓ The **Toolbar** tab is selected by default.

1. Click on an entry in the **Available Toolbar Items** list.
   → You will see a list of all available items in this group.
2. Double-click on an item.
   → The item will be added to the **Selected Toolbar Items** list and does appear in the **Toolbar** within the application. Alternatively you can add the items per Drag&Drop.
3. In order to change the order of symbols in the toolbar use the **Up**/**Down** buttons.
4. If you want to delete an Icon from the toolbar, click on the **Delete** button.
5. Click on **Close** button to close the dialog. The changes will be effective right now.

You have successfully customized the **Toolbar**.

11.3 Adjusting the Workspace Zoom

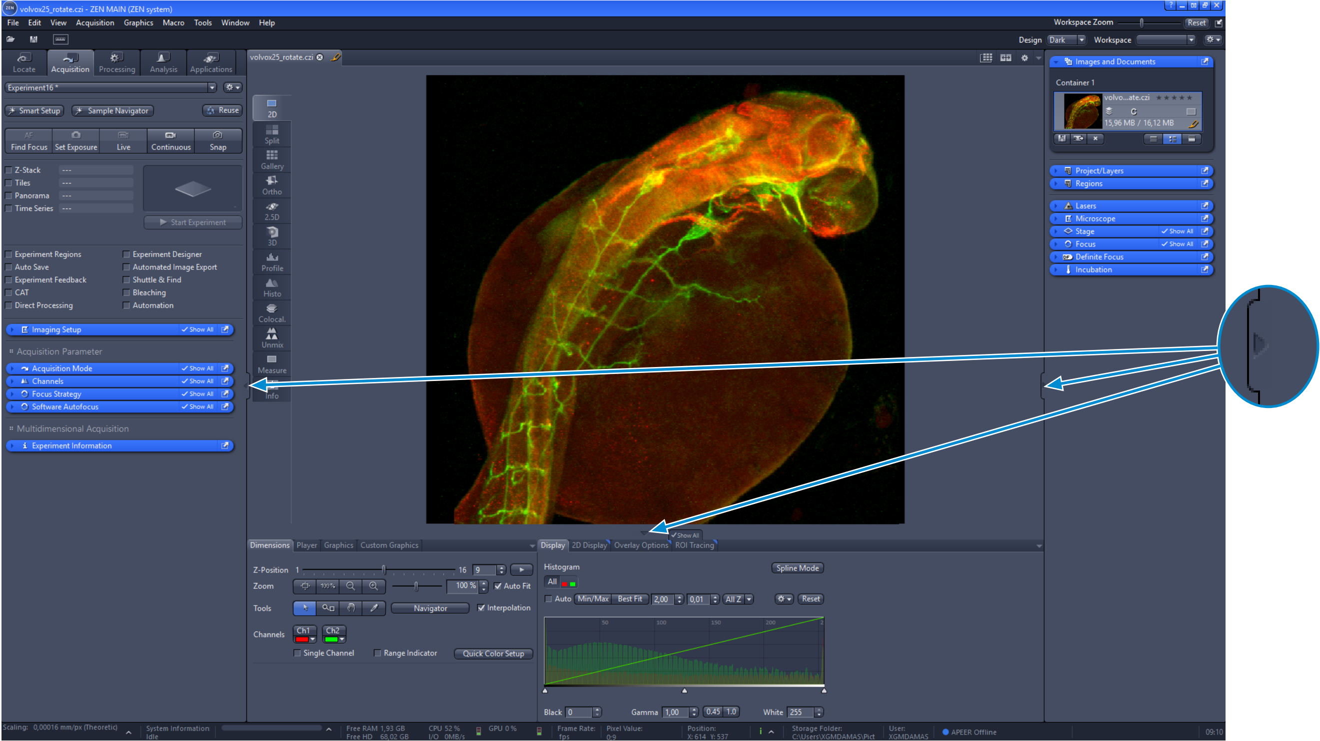
1. To zoom in or out of the workspace move the slider left or right.



2. To reset workspace zoom to default click on **Reset** button.

11.4 Showing/Hiding Workspace Areas

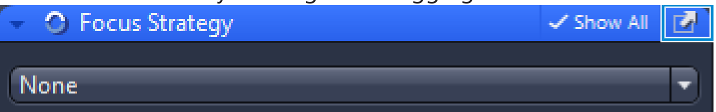
1. Click on **show/hide** buttons to show or hide areas.



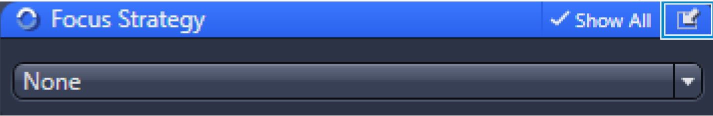
11.5 Undocking/Docking Tool Windows

This function allows you to undock/dock a tool window. An undocked tool window can be positioned anywhere on the screen.

1. Click the **Undock** button to undock a tool window. Once undocked, the tool window can be moved around by clicking and dragging it on the blue bar.



2. Click the **Dock** button to dock a tool window back to its place in the left tool area.



Info

With the **dock all tools** function in the Workspace Configuration you can globally attach all undocked tool windows back to the **Left Tool Area**

12 Software Modules & Extensions

12.1 Advanced Processing

12.1.1 Experiment Feedback

Experiment feedback (also known as conditional or adaptive experiments) allows you to define specific rules and actions to be performed during the acquisition of an experiment. It is possible to change the course of an experiment depending on the current system status or the nature of the acquired data during acquisition. Moreover, it is possible to integrate certain tasks like data logging or starting of an external application, directly into the ZEN experiment. A typical use-case is to connect the image acquisition with automated image analysis.

Feedback experiments can be set up and controlled with the *Experiment Feedback Tool [▸ 736]* and the *Script Editor [▸ 737]*. Experiment feedback functionality is a part of the **Advanced Processing** module. For an example workflow for experiment feedback, see *Workflow Experiment Feedback [▸ 240]*.

Please note that we will not describe experiment feedback in detail here, as you will find a detailed instruction on how to perform feedback experiments and a lot of tutorials on the latest ZEN DVD in the folder ...**...Manuals/ZEN(blue edition)/OAD Content/Experiment Feedback**.

Key Features

- Create smart experiments and modify the acquisition on-the-fly based on online image analysis, hardware changes or external inputs (e.g. TTL signals).
- The adaptive acquisition engine allows modifying running experiments according to the rules defined inside the feedback script.
- The feedback script uses ZEN commands in combination with the Python programming language
- The feedback script gives access to the current system status and results from the online image analysis during runtime of an experiment.
- Data Logging or starting an external application (e.g. Python, Fiji, MATLAB, . . .), directly from within the imaging experiment is possible.

12.1.2 Workflow Experiment Feedback

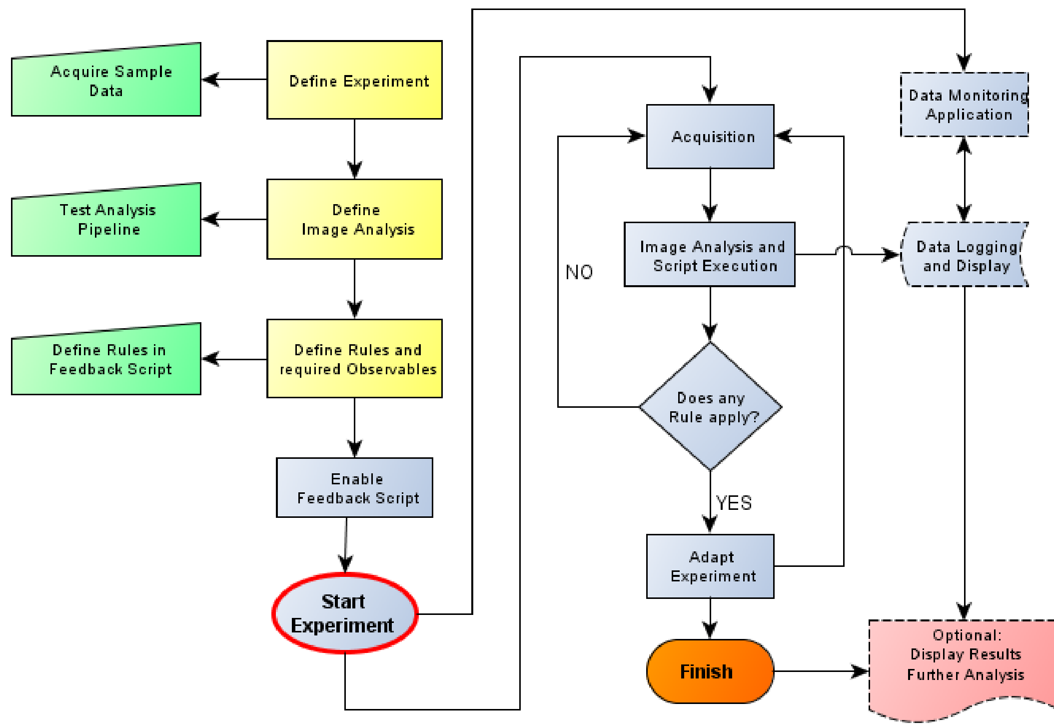


Fig. 12: Workflow Experiment Feedback

In the figure you can see the typical workflow of an **Experiment Feedback** experiment and the most important steps involved. The yellow boxes **Define Experiment**, **Define Image Analysis** and **Define Rules and Observables** show the most important steps for setting up a feedback experiment.

Step	Description
Define Experiment	Set up and configure the actual image acquisition experiment to obtain the desired image data, e.g. time lapse, Z-stack, multi-channel, tile acquisition etc. Once the setup of the acquisition is completed, you acquire sample data which will be used in the following step to setup and test the image analysis setting.
Define Image Analysis	Sets up an image analysis setting via the Image Analysis Wizard for the use inside the feedback script, if an analysis step is required. Only parameters specified in the image analysis setting can later be accessed from within the experiment feedback script. Test the image analysis setting to ensure the results of the image analysis are meaningful. For more information on the Image Analysis Wizard, see *Creating a new image analysis setting [▸ 369]*. For advanced analysis requirements it is also possible to use an OAD macro to create an image analysis setting (***.czias** file).
Define Rules and Observables	This step defines how the script actually works. Here, you define the rules in the feedback script, e.g. which parameters are observed and how the experiment should react when a certain event occurs.

Step	Description
Start Experiment	Start the **Experiment Feedback** experiment and watch the output. The general concept behind this workflow can be described as a loop, which is the actual acquisition. For every event, e.g. when a new image has been acquired, the script will be executed. The rules are checked and if required, certain tasks are carried out. Additionally, it is possible to log data into a text file and/or start an external application at any time point during the experiment.

See also

Script Editor for Experiment Feedback Dialog [737]

12.1.2.1 Editing the Feedback Script

To use the **Experiment Feedback** tool, you need to edit the feedback script.

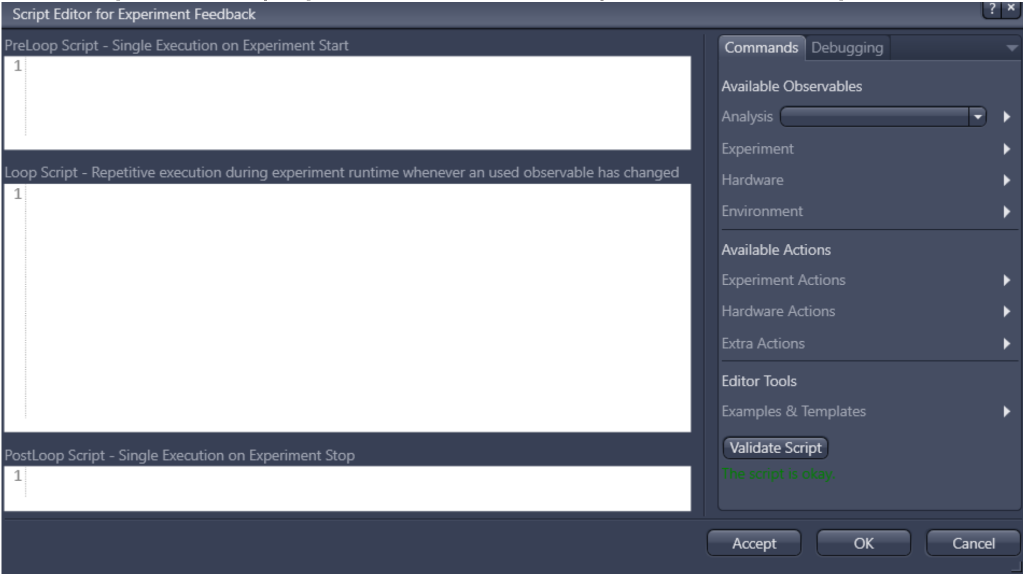
The main loop script run will only be triggered, when an observable that is used in the main loop script has changed. If the parameters within the loop script do not change, the script will not be executed.

These changes can be:

- A new image has been acquired
- The XYZ position has changed
- The status of a trigger port has been altered
- The settings for filter, objective, light source etc. have changed
- The incubation parameters have changed

For advanced use cases it is possible to execute the script with the command *RunLoopScript* in conjunction with a timer.

1. Select **Acquisition** tab **| Experiment Feedback** tool **| Edit Feedback Script**.



→ The **Script Editor for Experiment Feedback** dialog opens. For more information, see *Script Editor for Experiment Feedback Dialog [737]*

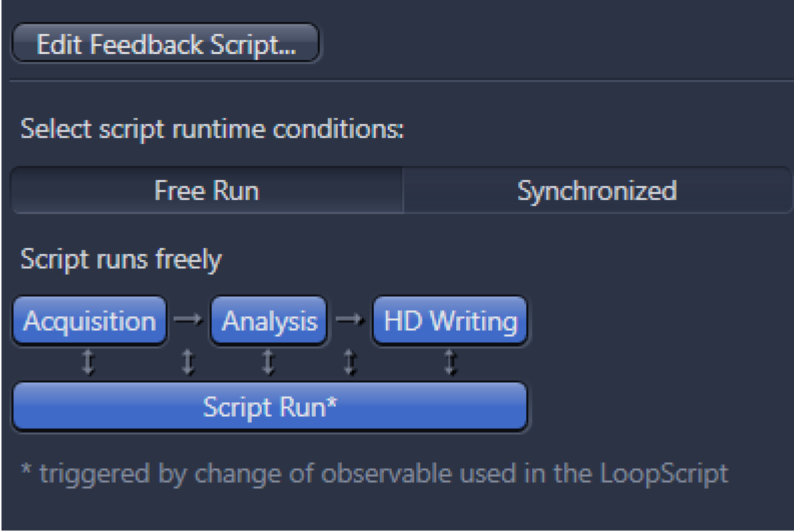
2. Create and edit the feedback script in the following sections: the **PreLoop Script**, the **Loop Script** and the **PostLoop Script**. To do so, use the commands on the **Command** tab. Add observables and actions to your experiment feedback script either via double-click or drag-and-drop. All observables and actions are also available via **IntelliSense auto-completion** starting with **ZENService**.
3. If an image analysis is part of your feedback experiment, you can select an existing image analysis you previously created. Select **Command** tab | **Available Observables** section | **Analysis** drop-down menu.
   → A list of the features that were defined in this image analysis setting, e.g. number of cells detected, is displayed. If you selected an image analysis setting and use one or more features in the feedback script, this image analysis setting is executed for each acquired image. Note that only features you have previously defined in the **Image Analysis Wizard** are available from within the Feedback Experiment. If you make any changes within the image analysis wizard to an existing *.czias file you need to reload it in the feedback script editor to activate the changes in the feedback experiment.
4. Click **OK**.
   → The existing code is validated. You can only save and close the script editor if the code is free from syntax mistakes.

The feedback script will be stored as part of the experiment file ***.czexp**.

12.1.2.2 Selecting script runtime conditions

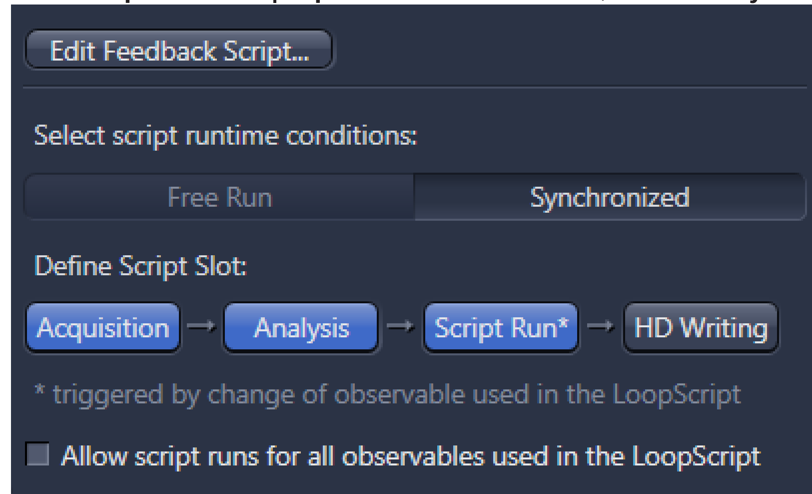
The script runtime conditions describe how the different steps of the experiment **Feedback Script** are executed. You can choose between **Free Run** and **Synchronized** execution.

1. Select **Acquisition** tab **| Experiment Feedback** tool, and select **Free Run**.



   → The execution of all steps of the Feedback experiment are independent and not executed sequentially.

1. Select **Acquisition** tab **|** **Experiment Feedback** tool, and select **Synchronized**.

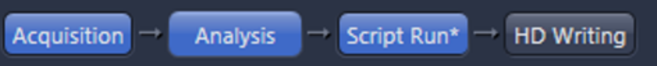


2. Click on the blue buttons to define the script slot and to change the execution order of the script.

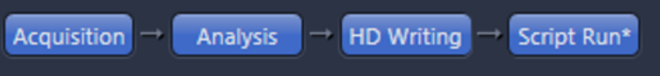
   → The selected steps are executed sequentially.

- The script run starts after the acquisition together with the online image analysis. Therefore, the script execution, image analysis and writing of the image subblock to the hard drive are not in sync.

- The online image analysis starts after the acquisition of a frame is finished. Only when the image analysis is finished, the next script run is triggered. This guarantees that all analysis results exist and can be used in the feedback script. The writing to disk is not synchronized.



- The online image analysis starts after the acquisition of a frame is finished. The image data are written to disk and only when all tasks are finished the next script run is triggered. This guarantees, that all analysis results exist and the image data is stored on disk before the next script run is triggered. This option is relevant in case the script starts an external application to analyze the data.



12.2 APEER (on-site)

This module enables you to execute modules of the APEER platform with ZEN (blue edition). APEER is an online platform to create customized workflows for image processing tasks of your microscopy images. For more information, see https://www.apeer.com. This module for ZEN (blue edition) is only available with the corresponding license.

12.2.1 System Requirements

To use the **APEER on-site** module locally on your Windows PC, your system needs to fulfill the following requirements to run the Docker software:

- Windows 10 64-bit: Pro, Enterprise, or Education (Build 16299 or later)
- Hyper-V and Containers Windows Features must be enabled (for more information, see also https://techcommunity.microsoft.com/t5/itops-talk-blog/step-by-step-enabling-hyper-v-for-use-on-windows-10/ba-p/267945)
- BIOS-level hardware virtualization support must be enabled
- 64 bit processor with Second Level Address Translation (SLAT)
- 4GB RAM

These are the system requirements of Docker (as of September 2020) and they are subject to change. For the current system requirements of Docker, please refer directly to the information page for the Docker installation (https://docs.docker.com/docker-for-windows/install/).

System Performance Disclaimer

To use APEER on-site, Hyper-V needs to be enabled in Windows. Please be aware that this might affect the overall PC system performance and, therefore, could lead to longer processing times or to reduced acquisition performance in ZEN (blue edition). If you encounter performance issues it is recommended to turn off Hyper-V again. The ideal case would be to use **APEER (on-site)** on a dedicated, powerful workstation that is not directly attached to a microscope. Enabling the BIOS level hardware virtualization did not influence the system performance and can be left switched on.

Hardware Recommendations:

- ZEISS MidRange Workstation
- 64 GB RAM or more (32 RAM dedicated to Docker Desktop)

12.2.2 Preliminary Work / Prerequisites

Following prerequisites need to be fulfilled to use this module:

- **Create an APEER Account**
  In order to use the functionality and download the APEER modules, you need an account on the platform. Go to https://www.apeer.com to sign up.
- **Install Docker**
  To be able to use your APEER modules locally on your machine, you also have to install the software called Docker (https://www.docker.com) which automates the deployment of code inside software containers. For installation instructions on a Windows PC, see https://docs.docker.com/docker-for-windows/install. You then have to manually allow access to your hard drive. After the installation, start Docker and go to **Settings > Shared Drives**. There you have to activate the checkbox for your hard drive (**C**) and click on **Apply**.

- **Set up a remote Linux machine**
  If you want to execute the APEER modules on a remote computer, you have to set it up. Currently only Linux machines running Docker are supported for the remote execution. For information on how to set up such a computer, please ask your local IT administration/department.
- **Create a shared folder**
  If you want to execute the APEER modules on a remote computer, you additionally have to create a folder to which both computers have access to over the network.

12.2.3 Creating and entering an API key

For the **APEER** modules, you also need an API key. Take the following steps to create the key and enter it in ZEN.

Prerequisite
- ✓ Your PC has a connection to the internet and you are signed in on the APEER platform.
- ✓ You have started ZEN.

1. Click on **Tools > Options > APEER**.
2. Click on the link above the input field (https://www.apeer.com/app/user/client-keys).
   → In your browser, the page for your personal access tokens opens.
3. Click on **New Access Token** to create a new access token.
4. In the **New Access Token** window, enter a name, an expiration date, and select the desired access scopes for the token.
5. Click on **Next**.
   → Your personal access token is created and displayed.
6. Copy your **Personal token**.
7. Go back to ZEN to **Tools > Options > APEER** and paste the token into the input field.
8. Under **Default Execution Location**, click on .
9. Select a location where the module is executed on your computer and your results are saved and click on **OK**.
10. Click on **OK** to apply the settings and close the **Tools > Options** window.

You have now created and entered an access token. You can now use APEER functionality in ZEN.

12.2.4 Setting up the remote module execution

If you want to execute the APEER modules on a remote Linux machine, you have to connect ZEN to this computer.

Prerequisite
- ✓ Your PC is connected to the network with the remote computer.
- ✓ You have set up a folder to which both PCs have access.

1. Go to **Tools > Options > APEER**.
2. In the **Choose Execution Mode** dopdown, select **Use Remote Docker Host**.
3. For **Remote Docker Host API Address** enter the address and port number of the remote computer.
4. Click on **Check Connection**.
   → ZEN contacts the remote computer with the given address and displays a green checkmarks if the connection was successful. Otherwise a red **x** is displayed and you have to correct the address and/or port information for the remote computer.
5. If your remote computer requires an authentication, select it from the **Choose Authentication Mode** dropdown and provide the required credentials (username and password or a certificate, depending on the selected mode).

6. Map the folder path for the execution of the module and saving of results on your local computer to the path of the same folder on the remote computer. For detailed information, see *Create a path mapping [▸ 246]*.
7. If you want to save the settings, click on **Export Remote Settings**, select the location in the file browser, and click on **Save**.
8. Click on **OK** to save the setup and close the dialog.

You have successfully set up the remote execution. Your modules are now executed on the selected remote computer. For more information, see *Executing APEER modules in ZEN [▸ 247]*.

See also

APEER Tab [▸ 642]

12.2.5 Create a path mapping

To execute an APEER module on your remote computer, you have to have a folder to which both PCs have access to. You then have to tell ZEN which folder it is and what the corresponding directory for this very same folder looks like for the remote computer. Since currently only Linux machines can be used for the remote task, the file systems on both PCs are different and the folder paths need to be mapped to one another.

Prerequisite

- ✓ You have set up a folder to which both PCs have access.
- ✓ You are in **Tools > Options > APEER**.
- ✓ For the **Choose Execution Mode** dopdown, **Use Remote Docker Host** is selected.

1. In the **Local To Remote File System Mapping** section, click on .
   → The *Edit Mapping Dialog [▸ 643]* opens.
2. For **Local Computer Path**, click on ... .
   → A file browser opens.
3. Select the shared folder to which both PCs have access to and click on **OK**.
4. For **Remote Computer Path**, enter the path to the same folder on the remote computer. If you do not know the directory path, please contact your IT department or person who set up the remote computer.
5. Click on **Check Mapping** to see if the two directories are correctly pointing to the same folder.
   → ZEN contacts the remote computer with the given address and displays a green checkmarks and corresponding message if the connection was successful. Otherwise a red **x** is displayed and you have to correct the path mapping.
6. Click on **OK**.

You have successfully mapped the folder path of your local computer to the one on the remote computer. You can now (continue to) set up the remote execution, see *Setting up the remote module execution [▸ 245]*.

12.2.6 Downloading an APEER module

Prerequisite

- ✓ You have done all the preliminary work and created an APEER API key. See also *Creating and entering an API key [▸ 245]*.
- ✓ Your PC is connected to the internet.
- ✓ You have started ZEN.

1. On the **Applications** tab, open the **APEER On-Site: Module Manager** tool.

2. Click on **Download Modules from APEER**.
   → A browser window opens.
3. In the browser window, select the module(s) you want to download and click on **OK**.
   → The download of the module(s) starts.

After a successful download the module is displayed in the **APEER On-Site: Module Manager** tool. It can now be used in ZEN.

See also

- APEER On-Site: Module Manager [▸ 249]
- APEER (on-site) [▸ 244]

12.2.7 Executing APEER modules in ZEN

Prerequisite

- ✓ You have downloaded an APEER module. For more information, see *Downloading an APEER module [▸ 246]*.
- ✓ If you execute the module on a remote computer, both PCs have to be connected via network and have access to the defined shared folder. See also *Setting up the remote module execution [▸ 245]*.

1. On the **Applications** tab, open the **APEER On-Site: Module Execution** tool.
2. For module, select the module you want to use locally from the dropdown list.
3. If applicable, you can then select which **Version** of the selected module you want to use.
   → The **Module Parameters** of the selected module are displayed.
4. Under **Module Parameters**, set the your parameters.
5. If your module supports multiple inputs and you want to use the module on more than one image, activate the checkbox **Use Batch Mode**.
6. Under **Module Input**, click on ... and select the input image(s) which should be processed by the module.
7. Select the **Execution Settings Location** where the module is executed on your computer and your results are saved.
8. Click on **Execute Module**.
   → Your selected APEER module is now executed.
9. To check the status and see the last executed modules, can click on **Browse Results** to open the *APEER Executions Browser [▸ 250]*.

You have successfully executed an APEER module.

See also

- APEER On-Site: Module Manager [▸ 249]
- APEER On-Site: Module Execution [▸ 250]

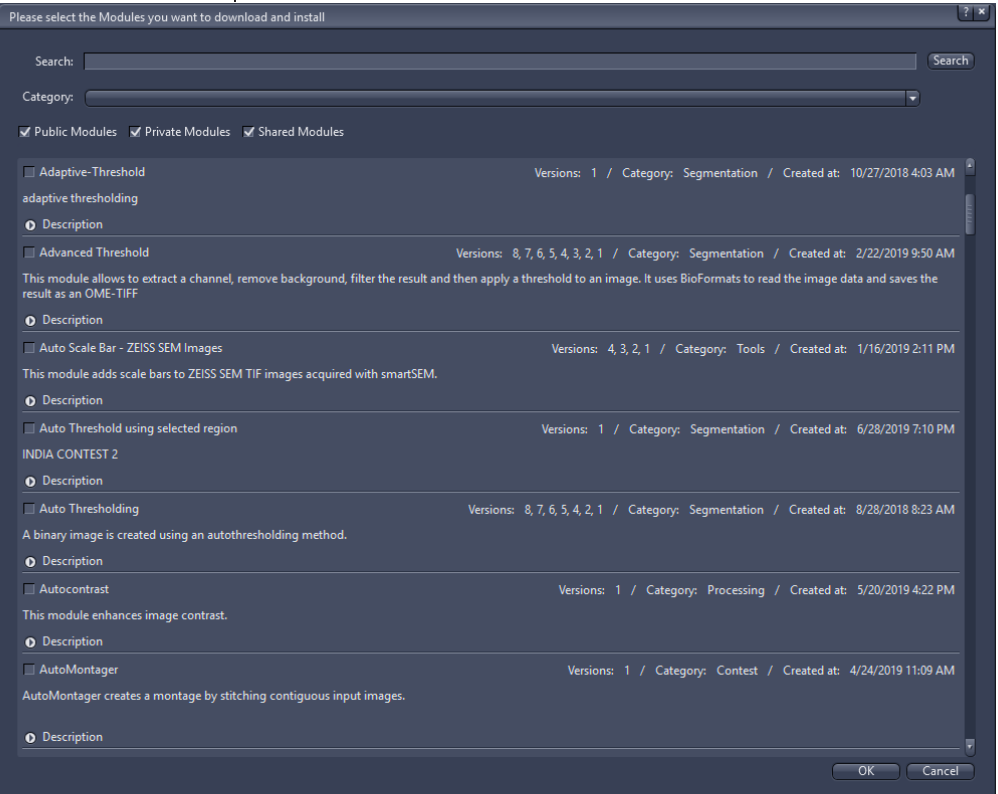
12.2.8 Example for APEER on-site

This chapter shows you how to download an APEER module and use it locally. For the purpose of this example, the **Auto Thresholding** module is downloaded and used locally.

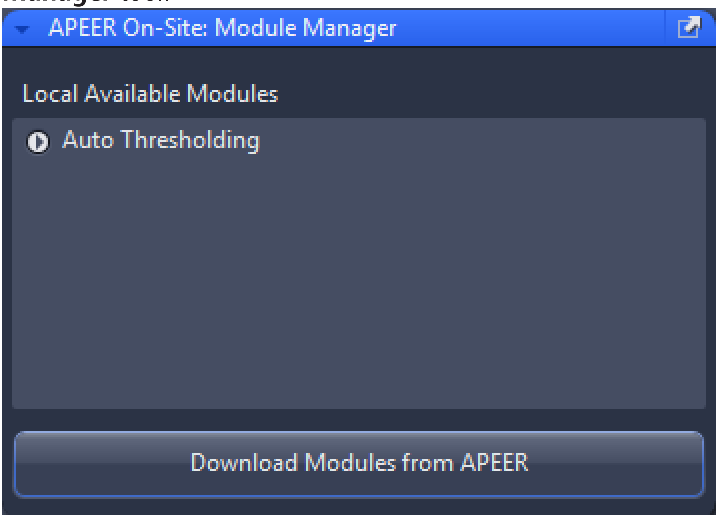
Prerequisite

- ✓ Your PC is connected to the internet.
- ✓ You have started ZEN.
- ✓ You have created an API key and entered it in **Tools > Options > APEER**. For more information, see *Creating and entering an API key [▸ 245]*.

1. On the **Applications** tab, open the **APEER On-Site: Module Manager** tool.
2. Click on **Download Modules from APEER**.
   → A browser window opens.

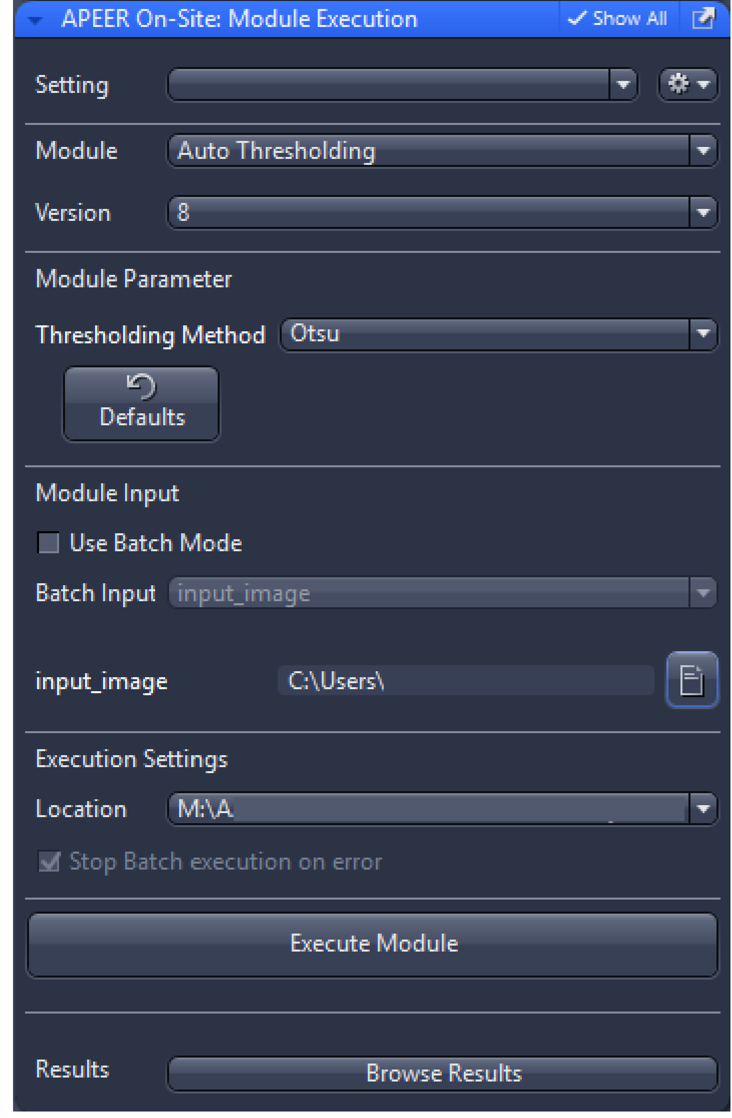


3. Select **Auto Thresholding** and click on **OK**.
   → The download of the module starts.
   → After a successful download the module is displayed in the **APEER On-Site: Module Manager** tool.



4. On the **Applications** tab, open the **APEER On-Site: Module Execution** tool.
5. In the Module dropdown list, select **Auto Thresholding**.

→ The parameter of the module **Auto Thresholding** are now displayed in the tool.



6. If applicable, you can change the **Version** of the module.
7. If you want to use the module on multiple images, activate the checkbox **Use Batch Mode**.
8. For **input_image**, click on [...] and select your input image(s).
9. For **Execution Settings Location**, select a location where the module is executed on your computer and your results are saved.
10. Click on **Execute Module**.

You have now downloaded the APEER module **Auto Thresholding** and executed it with ZEN.

12.2.9 Functions & Reference

12.2.9.1 APEER On-Site: Module Manager

Parameter	Description
Local Available Modules	Displays a list with all the downloaded and locally available APEER Modules. Click on [▶] to display a short description of the respective module and to have the possibility to delete it by clicking on [🗑].

Parameter	Description
Download Modules from APEER	Opens a browser window to download APEER modules. For more information, see also *Downloading an APEER module [▸ 246]*.

See also

APEER (on-site) [▸ 244]

12.2.9.2 APEER On-Site: Module Execution

Parameter	Description
Module	Selects the module you want to execute locally. Displays all downloaded modules in a dropdown list.
Version	Sets which version of the currently selected module should be executed.
Module Parameter	Displays and sets all the parameters of the currently selected module.
Module Input	Selects the image(s) for which the module is executed.
– Use Batch Mode	Only available for modules that support multiple input images. **Activated:** Enables the selection of multiple input images. **Deactivated:** The input can only select one image.
– Batch Input	Only available for modules that support multiple input images and if **Use Batch Mode** is activated. Selects the input for Batch execution.
input_image	Selects the input with a click on [...].
Execution Settings Location	Sets the path where the execution is located and where the results are saved.
Stop Batch execution on error	Only available for modules that support multiple input images and if **Use Batch Mode** is activated. **Activated:** Stops the Batch execution if an error occurs.
Execute Module	Executes the selected module.
Browse Results	Opens the *APEER Executions Browser [▸ 250]*.

See also

APEER (on-site) [▸ 244]
Executing APEER modules in ZEN [▸ 247]

12.2.9.3 APEER Executions Browser

This browser displays the last executed modules with detailed information.

Parameter	Description
Filter	
– Client or Username contains	Filters the executions that contain the characters entered in this field as part of the client and/or username.

Parameter	Description
– Status	Filters the executions that have the status that is selected from this dropdown list.
– Update Results	Updates the list of executions according to the filters.
List of Executions	
– Module Name	Displays the name of the executed module.
– Start	Displays the start date and time.
– Last Update	Displays date and time of the last update.
– Status	Displays the status of this execution.
– Client / User-name	Displays the name of the client and user that started the execution.
– Open Results	Opens a file browser at the location the results are saved.
– Open Log	Open the log file for this execution.
– Details	Opens the *Details for APEER Execution Dialog [▸ 251]*.

12.2.9.3.1 Details for APEER Execution Dialog

Displays details for this APEER execution.

Parameter	Description
Module Name	Displays the name of the executed module.
Module Version	Displays the version of the executed module.
Start Date	Displays the start date and time.
Last Update	Displays date and time of the last update.
Current Status	Displays the current status of the execution.
Starting Client	Displays the name of the client that started the execution.
Starting User	Displays the name of the user that started the execution.
Results Directory	Displays the directory where the results are saved.
Status History	Displays the status history with date and time for each status the execution had.
Ok	Closes the dialog.
Open Results	Opens a file browser at the location the results are saved.
Open Log	Open the log file for this execution.

12.3 APEER Workflows (online)

With this extension you can connect the ZEN software with the APEER platform (https://www.apeer.com). It provides the following functionality:

- Connect ZEN software to the APEER platform
- Start workflows (including upload of the current image)
- Optional: Download of workflow results

The current version of the APEER Workflows (online) is deployed together with the software. You will find additional information also on the APEER Wiki under https://docs.apeer.com/ and the APEER user forum under https://forum.apeer.com/.

Prerequisites

- **Create an APEER Account**
  In order to use the functionality and download the APEER modules, you need an account on the platform. Go to https://www.apeer.com to sign up.
- **API key**
  To use the functionality in ZEN, you need an API key. For detailed information, see *Creating and entering an API key [▸ 245]*.

12.3.1 Starting Workflows

Prerequisite

- ✓ You have acquired an image.
- ✓ You have created an API key and entered it in ZEN. For more information, see *Creating and entering an API key [▸ 245]*.

1. On the **Processing** tab, select the APEER image processing function **Start Workflow**.
2. In the **Input** tool, select an image as an input for the workflow. Note that the desired images must be opened in ZEN.
3. In the **Parameters** tool, select an APEER workflow from the dropdown list under **Workflow**.
4. If you want to download the workflow results to your local computer, activate the checkboxes **Wait for workflow finished** and **Download workflow results**.
5. If you want that the workflow results are automatically opened in ZEN, activate the checkbox **Display workflow results**.
6. Click on **Apply** to execute the workflow.

12.3.2 Uploading Files

Prerequisite

- ✓ You have acquired an image.
- ✓ You have created an API key and entered it in ZEN. For more information, see *Creating and entering an API key [▸ 245]*.

1. On the **Processing** tab, select the APEER image processing function **Upload Files**.
2. In the **Input** tool, select the image for the upload. Note that the desired images must be opened in ZEN.
3. Click on **Apply** to upload the selected image.

12.3.3 Functions & Reference

12.3.3.1 Start Workflow

With this image processing function you trigger a workflow on the APEER platform from ZEN. For more information, see *Starting Workflows [252]*.

Parameter	Description
Workflow	Selects a workflow from the APEER platform.
Wait for Finished Workflow	**Activated:** ZEN waits until the workflow is finished on the APEER platform.
Download Work-flow Results	Only available if **Wait for workflow finished** is activated. **Activated:** Downloads the workflow resultss from the APEER plat-form to your local computer.
Display Workflow Results	Only available if **Download workflow results** is activated. **Activated:** Displays the workflow results in ZEN after they have been downloaded from the APEER platform.
Storage	Sets the storage location for the download of the workflow results.

See also

APEER Workflows (online) [252]

12.4 Automated Photomanipulation

This module is exclusively available for the **Celldiscoverer 7** and allows automated photoactiva-tion and bleaching at multiple positions. It is not applicable to Tile Regions. Using this module, the system executes the following experiment steps without user interaction:

- Acquisition of a multi-position image as defined in the *Tiles Tool [531]*.
- Identification of the photomanipulation ROIs based on a customized image analysis that was defined beforehand in the *Image Analysis Wizard [777]*.
- Photomanipulation experiment as defined for Bleaching and in the *Time Series Tool [732]*.

For Automated Photomanipulation you need the license for the Automated Photomanipulation module and the Image Analysis license. The module is activated in **Tools > Modules Manager > Optional Software** and the tool for the model is displayed on the **Applications** tab.

12.4.1 Using Automated Photomanipulation settings

Automated Photomanipulation offers you the possibility to save your whole experiment setup in a settings file.

Creating an Automated Photomanipulation setting

1. On the **Applications** tab, open the **Automated Photomanipulation** tool.
2. Click on Options and select **New**.
3. Name the setting and press *Enter* or click on .

You have created a setting for Automated Photomanipulation.

Saving an Automated Photomanipulation setting

When you have set up your Automated Photomanipulation experiment and created a setting, you can save the setup as a setting.

1. Click on Options and select **Save**.

Your experiment setup is now saved.

Importing and exporting an Automated Photomanipulation setting

1. On the **Applications** tab, open the **Automated Photomanipulation** tool.
2. Click on Options and select **Import** or **Export**.
   → A file browser opens.
3. Select the file you want to import or the folder where you want to export the setting to.
4. Click on **Open** / **Save**.

You have now imported/ exported a setting.

Deleting an Automated Photomanipulation setting

1. On the **Applications** tab, open the **Automated Photomanipulation** tool.
2. Select the setting you want to delete in the dropdown list.
3. Click on the Options and select **Delete**.
4. Confirm that you want to delete the file.

The selected setting is deleted.

See also

- Automated Photomanipulation Tool [▸ 255]
- Performing an Automated Photomanipulation experiment [▸ 254]

12.4.2 Performing an Automated Photomanipulation experiment

For a successful Automated Photomanipulation experiment, you have to prepare a photomanipulation experiment at multiple positions and an image analysis setting.

Prerequisite

- ✓ You have activated the Automated Photomanipulation module in **Tools > Modules Manager > Automated Photomanipulation**.
- ✓ You have defined and saved a suitable experiment for photomanipulation at multiple positions (including Tiles, Bleaching, and Time Series).
- ✓ You have defined experiment positions in the **Positions** section of the **Tiles** tool. For more information, see *Positions Section [▸ 535]*.
  Important: Photomanipulation with **Tile Regions** is not supported!
- ✓ On the **Acquisition** tab, you have selected if the photomanipulation should be executed at **All Tile Regions per Time Point** (e.g. for photoactivation) or as **Full Time Series per Tile Region** (e.g. for photobleaching).
- ✓ You have defined photomanipulation settings in the **Timed Bleaching** tool. For more information, see *Timed Bleaching Tool [▸ 762]*.
- ✓ You have defined a suitable image analysis setting using the Image Analysis Wizard or an OAD macro that detects the regions of interest where the photomanipulation should be executed. For more information, see *Creating a new image analysis setting [▸ 369]*.
  Important: The classes in the analysis and their corresponding channel names must exactly fit to the channel names in the experiment!

1. On the **Applications** tab, open the **Automated Photomanipulation** tool.
2. Create a setting to save your experiment setup. For more information, see *Using Automated Photomanipulation settings [▶ 253]*.
3. For **Experiment**, select the experiment you want to use for photomanipulation. For more information, see *Set up a new experiment [▶ 44]*.
   Note: You have to acquire a snap and draw one Experiment Region. **Bleaching** must be activated. This enables you to define the settings for **Timed Bleaching**. This Experiment Region is not used for the photomanipulation experiment.
4. For **Analysis**, select a suitable setting to analyze the multi-position image.
5. For **Class**, use the dropdown list to select the appropriate class / channel to identify the photomanipulation regions of interest.
6. For **Sorting Feature**, define the acquisition order of the ROIs for photomanipulation. This step depends on the selected features in the analysis (e.g. ID, mean intensity in channel x, perimeter etc.). Per default, the photomanipulation per position is executed in the descending order of the IDs.
7. For **Region Type**, define the ROI shape and maximum ROI number per position for photomanipulation.
8. For **Output Folder**, define the folder where you want to store the experiment data.
   The complete experiment including all positions and photomanipulation events is stored as one .czi file. This folder also contains the first scanned image for ROI identification (InitialAnalysisSettingImage.czi) and the table of the ROIs (SingleObjectsTable.csv).
9. Click on **Start**.
   → A InitialAnalysisSettingImage.czi image is acquired to identify the ROIs for photomanipulation and saved to your folder. The ROIs are listed in the SingleObjectsTable.csv table. They are automatically imported as Experiment Regions for photomanipulation.
   → Then the photomanipulation experiment at multiple positions is executed.
   → In the Mean ROI tab of the resulting .czi file, the bleach markers are shown.
   → To check the ROI selection, you can analyze the InitialAnalysisSettingImage.czi with the predefined analysis.
   → The **Start** button turns into a **Stop** button as long as Automated Photomanipulation is running. Click on **Stop** to stop the running Automated Photomanipulation workflow.

You have successfully performed a Automated Photomanipulation experiment.

12.4.3 Functions & Reference

12.4.3.1 Automated Photomanipulation Tool

Parameter	Description
Options	
– New	Creates a new Automated Photomanipulation setting. Enter a name for the setting.
– Rename	Renames the setting.
– Save	Saves the current setting.
– Save as	Saves the current setting under a new name. Enter a name for the setting.
– Import	Imports an existing setting. The setting is shown in the setting dropdown list.

Parameter	Description
– Export	Exports the current setting.
– Delete	Deletes the current setting.
Experiment	Selects the experiment setup to execute the photomanipulation including Tiles, Time Series and Bleaching.
Analysis	Selects the image analysis setting used to analyze the multi-position image to define the Experiment Regions for photomanipulation.
Class	Defines the class / parameter to find the photomanipulation experiment regions. **Important:** The classes in the analysis and their corresponding channel names must exactly fit to the channel names in the experiment.
Sorting Feature	Defines the acquisition order of the Experiment Regions for photomanipulation depending on the selected features in the analysis (e.g. ID, mean intensity in channel x, perimeter etc.).
Region Type	Defines the shape of the photomanipulation area: Polygon, Circle, Rectangle, Custom Circle/Rectangle (Size and Offset adjustable), Circle, and Rectangle are bounding / containing the detected photomanipulation regions.
Max. Number	Defines how many photomanipulation ROIs per position should be executed.
Offset	Only visible if **Custom Circle** or **Custom Rectangle** is selected. Defines the Experiment Regions offset of the Custom Circle/Rectangle in X and Y direction.
Size	Only visible if **Custom Circle** or **Custom Rectangle** is selected. Defines the Experiment Regions size of the Custom Circle/Rectangle in width and height.
Output Folder	Selects the folder where the experiment results are saved. A subfolder will automatically be created for each run of an Automated Photomanipulation experiment, including the pre-bleach image for Experiment Regions identification, the table of all Experiment Regions, and the experiment results.
Start	Starts the Automated Photomanipulation. The experiment can be stopped with the **Stop** button in the **Acquisition** tab. Click on **Stop** to stop the running Automated Photomanipulation workflow.

See also

Automated Photomanipulation [▸ 253]

12.5 Confocal Topography

This module includes the following functionality:

Topography Acquisition

With the **Topography Tool** you can acquire and inspect surfaces or surface structures of different sample types (e.g. wafer plates, solar cells). By the help of the **Topography Measurement Wizard** you can perform an confocal image acquisition of your sample. The advanced analysis of the topography image is performed by the help of the ConfoMap software which is included in the software package.

In the chapter **Functions & Reference** you find detailed functional descriptions of the *Topography Tool [▸ 265]* and the *Topography Measurement Wizard [▸ 265]*.

In the chapter **Workflow Topography Acquisition** you find a detailed how-to guide for topography acquisition, see Introduction.

Layer Thickness Measurement

With the **Layer Thickness Measurement Tool** you can acquire z-stacks and perform layer thickness measurements of one or more transparent layers (e.g. coatings, alloys). Here as well, the **Layer Thickness Measurement Wizard** will guide you through the process. The measured values can be corrected by the refractive index of the coating.

In the chapter **Functions & Reference** you find detailed functional descriptions of the *Layer Thickness Measurement Tool [▸ 267]* and the *Layer Thickness Measurement wizard [▸ 267]*.

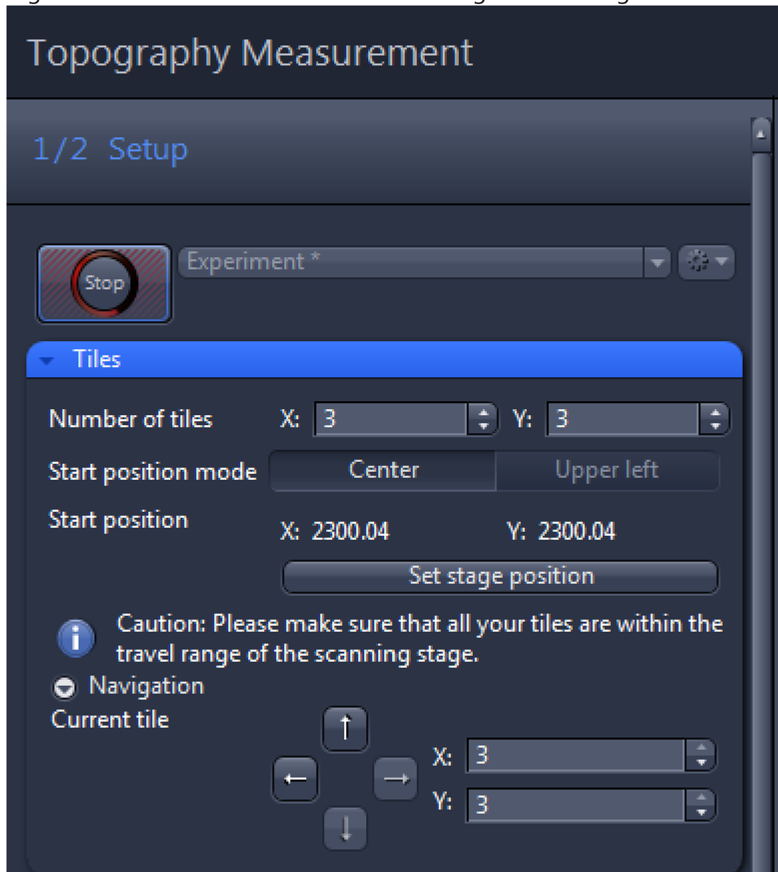
In the chapter **Workflow Layer Thickness Measurement** you find a detailed how-to guide for layer thickness measurement, see Introduction.

12.5.1 Acquiring Topography Images

If you want to acquire a topography image in ZEN, you need to start the topography measurement wizard.

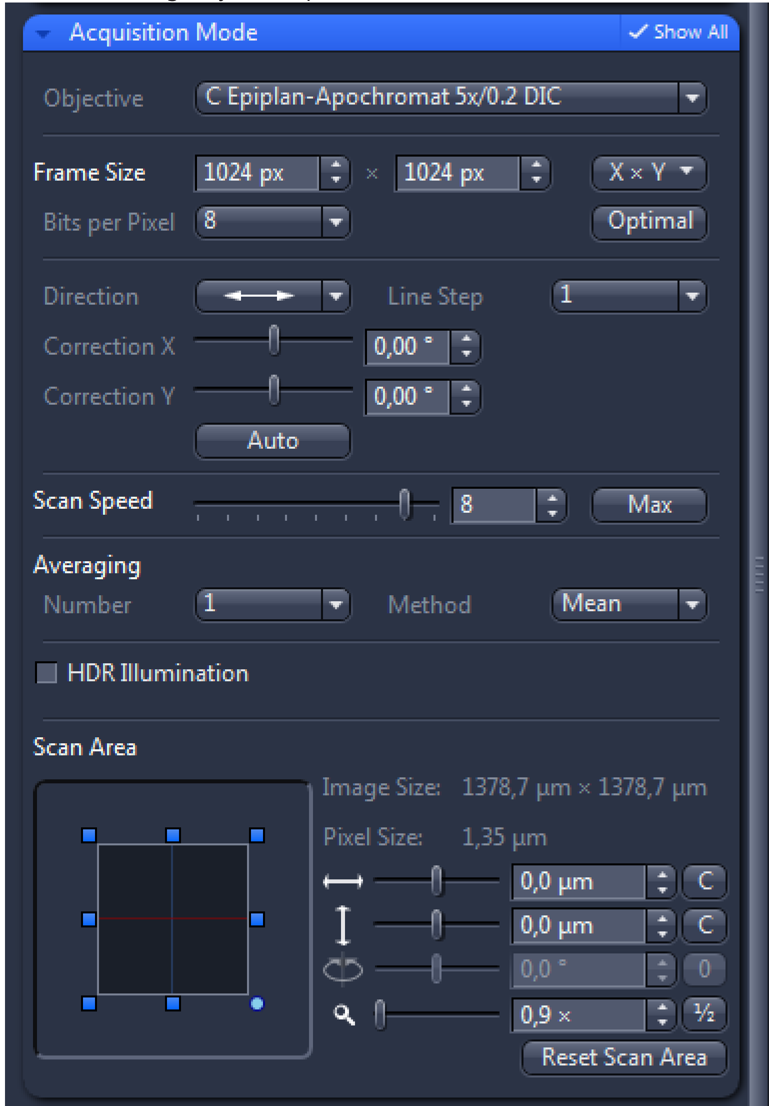
Prerequisite ✓ You are on the **Applications** tab.

1. In the **Topography** tool, click on **Start**.
   → The wizard starts. In the left tool area you see the Step 1/2 **Setup**, in which you can adjust the acquisition parameters for the topography image. In the center screen area you see the live image from your sample in continuous mode.
   As a default the system is set up to acquire only one single z-stack. In case your region of interest (ROI) is all within your field of view (FoV) continue with step 5 of this guide. If your ROI is bigger than your FoV, you can setup a tiles acqusition in the Tiles tool:
2. In the **Tiles** tool, enter the number of desired tiles in the **X** and/or **Y** direction input fields, e.g. 3 x 3. This will create a 3 x 3 tiles image with 9 single tiles.

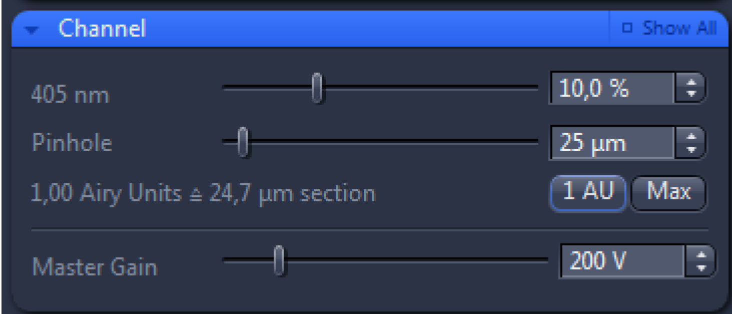


3. Click on **Set stage position** to define the anchor point of your tile scan at the current stage position.
4. In the **Navigation** (must be expanded first) section click on the arrow buttons to navigate through the single tile images. This can help to check if the tiles cover the full ROI.

5. In the **Acquisition Mode** tool, adjust the settings for **Objective**, **Frame Size**, and **Bits per Pixel** according to your requirements.

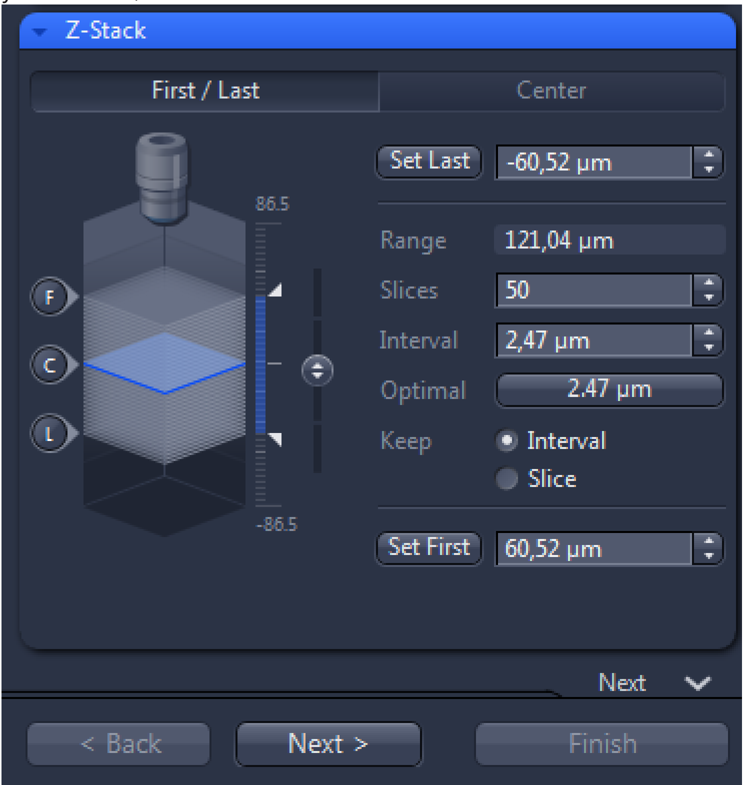


6. Focus on the surface. The default pinhole setting is **Max**. This allows you to easily focus on the surface just like with a widefield microscope. Of cause focusing via the eyepieces is also possible.
7. In the **Channel** tool, adjust the pinhole to the required diameter. For best performance of the system we recommend to set the pinhole size to 1 Airy Unit (**1AU** button). Since the depth of field is reduced in confocal images you most probably need to slightly adjust the focus position.



8. Adjust the intensity of the laser. To do so focus on the brightest section of the z-stack. Then adjust the laser either via the **405 nm** slider or the **Master Gain** slider. Adjust the intensity so that you do not see any overexposed pixels. (shown in red).

9. In the **Z-Stack** tool, set up the **Z-Stack** by adjusting the z-range (upper and lower limit of your z-stack). We recommend to use **First / Last** mode for that.



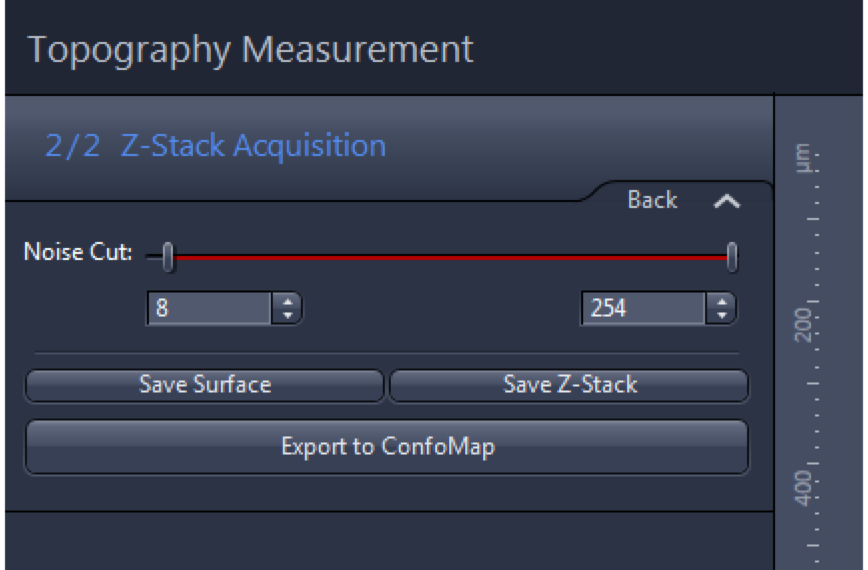
→ Make sure the whole surface, you want to measure, is within these limits. In case of a tile scan you can use the navigation arrows in the **Tiles** tool to jump to the different tiles to check.

10. Click on **Next**.

→ The wizard moves to **Step 2/2 Z-Stack Acquisition** and starts the image acquisition.

→ After image acquisition the topography image (height map) will be automatically generated. You will see the image in the center screen area after the acquisition and the processing is finished.

11. Adjust the thresholds by using the **Noise Cut** slider, if necessary. Note that pixels below the lower threshold are displayed in blue color and pixels above the upper threshold in red color.



12. Finish your work in ZEN by choosing the following options:
    - If you click on **Export to Confomap** the topography image will be directly opened in the **ConfoMap** analysis software.
    - If you click on **Save Topography** you can save the topography image to the file system.
    - If you click on **Save Z-Stack** you can save the raw data of the z-stack.

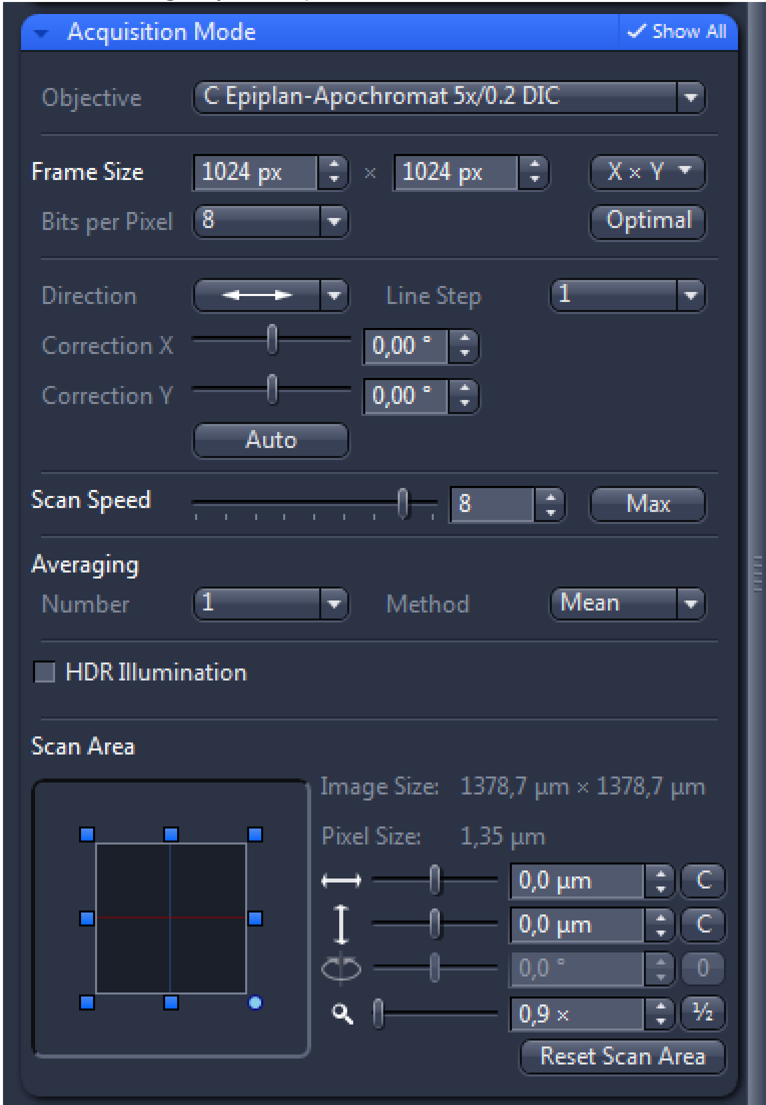
You have successfully acquired a topography image. As the image analysis is performed with ConfoMap software read the ConfoMap documentation for further information.

12.5.2 Measuring Layer Thickness

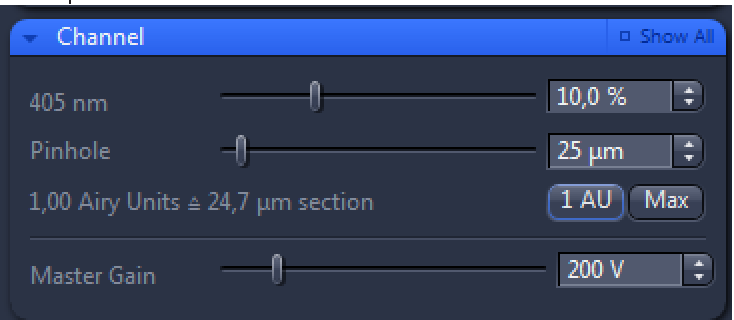
If you want to perform layer thickness measurement in ZEN, you need to start the topography measurement wizard.

Prerequisite ✓ You are on the **Applications** tab.

1. In the **Layer Thickness Measurement** tool, click on **Start**.
   → The wizard starts. In the left tool area you see the step **1/3 Setup**, in which you can adjust the acquisition parameters for the topography image. In the center screen area you see the live image from your sample in continuous mode.
2. In the **Acquisition Mode** tool adjust the settings for **Objective**, **Frame Size**, and **Bits per Pixel** according to your requirements.



3. Focus on the surface. The default pinhole setting is **Max**. This allows you to easily focus on the surface just like with a widefield microscope. Of cause focusing via the eyepieces is also possible.
4. In the **Channels** tool, adjust the pinhole to the required diameter. For best performance of the system we recommend to set the pinhole size to 1 Airy Unit (**1AU** button). Since the depth of field is reduced in confocal images you most probably need to slightly adjust the focus position.



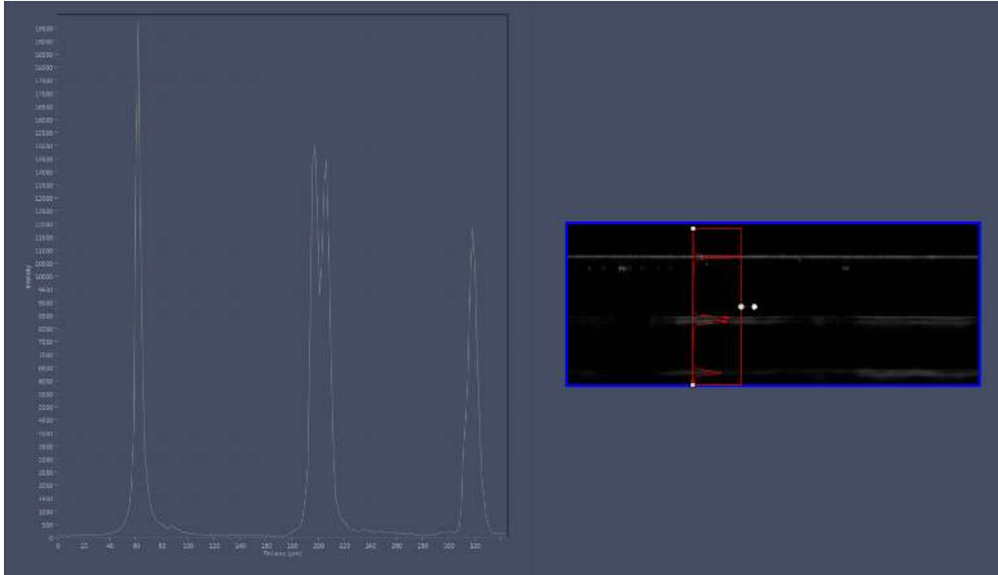
5. Adjust the intensity of the laser. To do so focus on the brightest section of the z-stack. Then adjust the laser either via the **405 nm** slider or the **Master Gain** slider. Adjust the intensity so that you do not see any overexposed pixels. (shown in red).
6. In the **Z-Stack** tool, define the z-stack by adjusting the z-range (upper and lower limit of your z-stack). We recommend to use **First / Last** mode for that.
   → Make sure that all layers, you want to measure, are within these limits. In case of a tile scan you can use the navigation arrows in the **Tiles** tool to jump to the different tiles to check.
7. Click on **Next**.
   → The wizard moves to step **2/3 Sectioning** and starts the image acquisition.
   → After image acquisition and processing you will see the image in the center screen area.
8. Click on **X-Z Layer** or **Y-Z Layer** to create the corresponding cross section which you want to analyze in detail.

9. Click on **Next**.
   → The wizard moves to step **3/3 Measurement**.

→ The selected cross section will appear on the right sight of the screen.

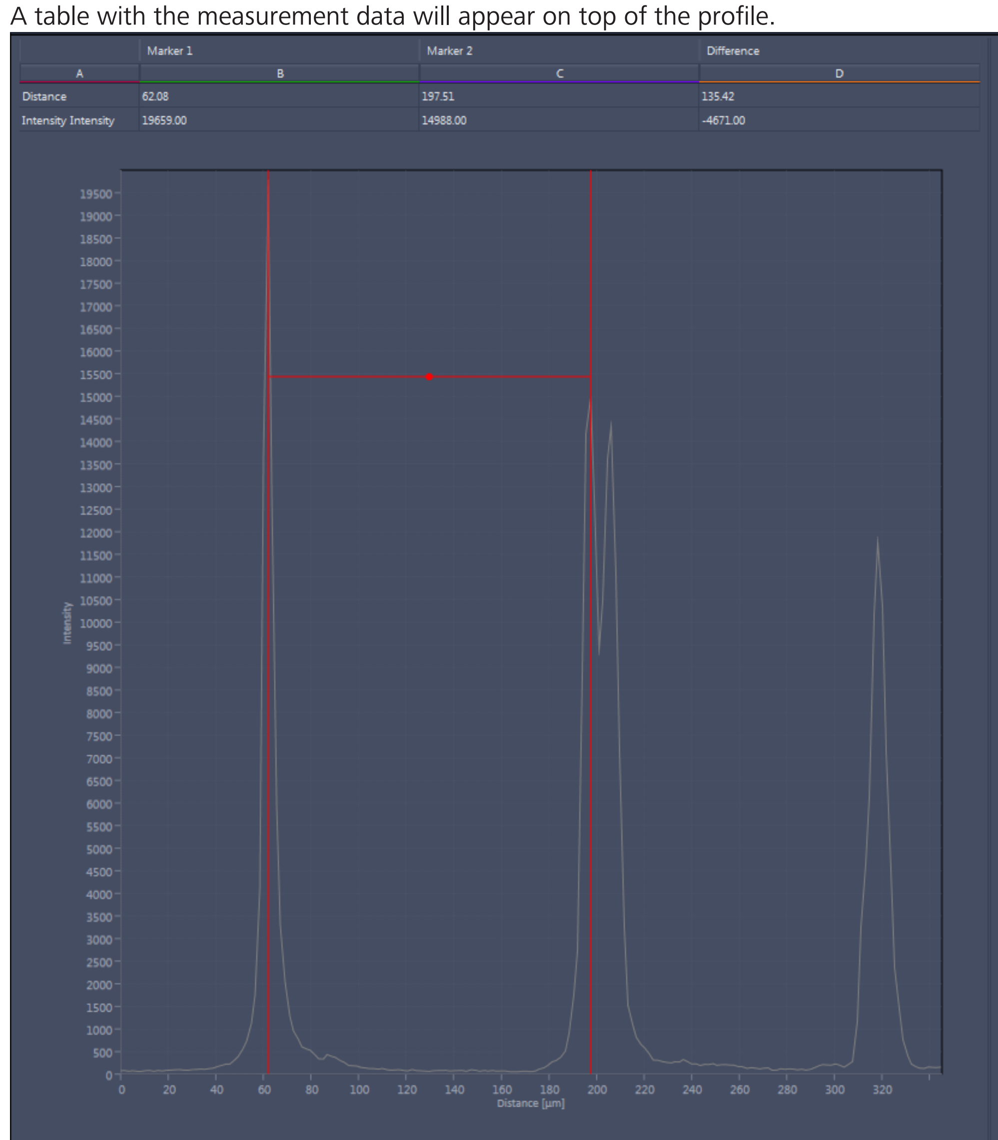
10. Draw in a profile perpendicular to your surface.

→ The intensity distribution of your profile will appear left to the cross section.

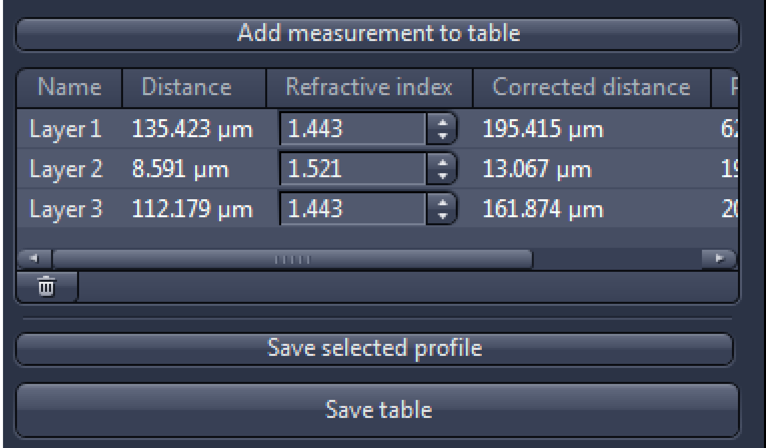


11. Position the calipers on the maximum intensity of the profile.

→ A table with the measurement data will appear on top of the profile.



12. Click on **Add measurement to table** to transfer the data to the result table on the left. Within the table you are able to rename the layers and calculate the true layer thickness by adding the refractive index.



13. Finally you have three options how to save your results:
    - If you click **Save table** the result table is saved on the file system.
    - If you click **Save selected profile** the drawn in profile is exported for further investigation in third party software.
    - If you click **Save Z-Stack** the raw data of the z-stack is saved on the file system.

You have successfully measured the layer thickness of your sample, saved the results table, the profiles and the raw data of the z-stack.

See also

Layer Thickness Measurement Wizard [▸ 267]

12.5.3 Functions & Reference

12.5.3.1 Topography Tool

Parameter	Description
Image Acquisition	If this option is selected you can acquire an image by the help of the **Topography Measurement** wizard. The wizard guides you through the image acquisition. Then you can export the image to **ConfoMap** for further processing. The wizard is started by the **Start** button at the bottom of the tool.
Load Z-Stack	If this option is selected, you can load an existing confocal z- stack from the file system. Therefore simply click on the folder icon and select the z-stack image from the file system. If you click the **Start** button, the **Topography** wizard will directly switch to the last wizard step to apply the noise cut and transfer the data to **ConfoMap**.
Automatic Noise Cut	If activated, the wizard will automatically perform the noise cut filtering with the preset parameters in the tool. If not activated, the noise cut filtering is an additional step in the **Topography Measurement** wizard.
- Thresholds	Here you can define thresholds for 8-bit and 16-bit images.
Start	If you click on this button, the *Topography Measurement Wizard [▸ 265]* is started.

See also

Layer Thickness Measurement Tool [▸ 267]
Layer Thickness Measurement Wizard [▸ 267]
Acquiring Topography Images [▸ 258]

12.5.3.2 Topography Measurement Wizard

When you have selected **Image Acquisition** in the **Topography** tool the **Topography Measurement** wizard consist of two steps:

Step 1/2: Setup

This step allows you to choose an experiment or setup imaging parameters for a new image acquisition, e.g. number of tiles, acquisition mode or z-stack creation, see *Step 1: Setup [266]*.

Note that when you have selected **Load Z-Stack** in the **Topography** tool and you start the wizard, this step is not available.

Step 2/2: Z-Stack Acquisition

The second step is Z-Stack Acquisition. The acquisition will be performed automatically. Additionally you can apply noise cut filtering. Pixels which are effected by the thresholds will be displayed in red (upper threshold) or blue (lower threshold), see *Step 2: Z-Stack Acquisition [266]*.

Info

The noise cut filtering will be skipped if the checkbox **Automatic Noise Cut** in the **Topography** tool is activated. The preset parameters will be immediately applied to the image and the wizard continues with the export to ConfoMap.

See also

- Topography Tool [265]
- Layer Thickness Measurement Wizard [267]
- Acquiring Topography Images [258]

12.5.3.2.1 Step 1: Setup

After the wizard is started, you will see the live image (Continuous mode) from your sample in the Center Screen Area. You can setup the acquisition parameters in the left area of the screen. In the next chapters the individual steps are described.

Note that these are default ZEN blue tools customized for acquisition of topography images, see chapter Acquisition Setup Tools.

See also

- Tiles [269]
- Channel [269]
- Acquisition Mode [270]
- Z-Stack [272]

12.5.3.2.2 Step 2: Z-Stack Acquisition

Parameter	Description
Noise Cut	Here you can adjust the noise cut parameters manually. Therefore use the slider or enter the lower and upper values in the input fields.
Save Surface	Save the image of the surface to the file system.
Export to ConfoMap	Exports the image to the ConfoMap software. The ConfoMap software will be started automatically after you have clicked the button.

12.5.3.3 Layer Thickness Measurement Tool

Parameter	Description
Image Acquisition	If this option is selected you can acquire an image by the help of the **Layer Thickness Measurement** wizard. The wizard guides you through the image acquisition. Then you can export the image to **ConfoMap** for further processing. The wizard is started by the **Start** button at the bottom of the tool.
Load Z-Stack	If this option is selected, you can load an existing confocal z- stack from the file system. Therefore simply click on the folder icon and select the z-stack image from the file system.
Start	If you click on this button, the **Layer Thickness Measurement** wizard (see *chapter > [267]*) is started.

See also

- Topography Tool [265]
- Topography Measurement Wizard [265]
- Measuring Layer Thickness [261]

12.5.3.4 Layer Thickness Measurement Wizard

When you have selected **Image Acquisition** in the **Layer Thickness Measurement** tool the **Layer Thickness Measurement** wizard consist of three steps:

Step 1/3: Setup

In this step you choose an experiment or setup the imaging parameters for a new z-stack acquisition e.g., track setup, acquisition mode, z-stack settings, see *Step 1: Setup [268]*.

Step 2/3: Sectioning

In this step (orthogonal sectioning) you define the cross section of the sample, you want to analyze. Here, in addition to the center view (**X/Y** axis; blue frame), you will also see the section views of the **X/Z** axes (top, green frame) and **Y/Z** axes (right, red frame), see *Step 2: Sectioning [268]*.

Step 3/3: Measurement

In this step you draw one ore more intensity profiles in your cross section. In these profiles you can measure and create tables containing the measurement results. Finally you can save the profile, the z-stack or the result table, see *Step 3: Measurement [268]*.

See also

- Layer Thickness Measurement Tool [267]
- Topography Measurement Wizard [265]
- Measuring Layer Thickness [261]

12.5.3.4.1 Step 1: Setup

After the wizard was started, you see the live image (Continuous mode) from your sample in the Center Screen Area. You can setup the acquisition parameters in the left area of the screen. In the next chapters the individual steps are described. Note that these are default ZEN blue tools customized for acquisition of layer thickness measurement images.

See also

- Channel [269]
- Acquisition Mode [270]
- Z-Stack [272]

12.5.3.4.2 Step 2: Sectioning

Here you can adjust the sectioning parameters.

Parameter	Description
X-Z Layer (green)	If selected the X-Z section will be used for further investigation.
Y-Z Layer (red)	If selected the Y-Z section will be used for further investigation.
Cut Lines	Here you adjust the position of the desired cut line. Therefore use the slider or directly enter the position in the input field.
Mid button	If you click on this button, the corresponding cut line will be positioned in the middle (center) of the image.
Line Width	Here you adjust the line width of the corresponding cut line. Default value is 1 (in pixel).

12.5.3.4.3 Step 3: Measurement

Here you can adjust the measurement parameters.

Parameter	Description
Profile mode	Here you can select the profile mode for the measurement:
- **Arrow**	If selected, you can draw in an arrow in the profile. This will measure the profile along the drawn in line of the arrow.
- **Rectangle**	If selected, you can draw in a rectangle to the profile. This will measure the profile in the whole area of the rectangle.
Add measurement to table	If you click on this button, the measurement result will be added to the measurement table at the left side. In this table you can rename your profile and correct the result by the refractive index.
Save selected profile	If you click on this button, the selected profile will be saved as .czt file.
Save Z-Stack	If you click on this button, the Z-Stack image will be saved as .czi file.
Save table	If you click on this button, the table will be saved as .czt file.

12.5.3.5 Tiles

Here you setup the tiles acquisition. The following parameters are available:

Parameter	Description
Number of Tiles	Here you can enter the desired number of tiles in X- and Y-direction.
Set stage position	If you click on this button, the current stage position is defined as starting position for the tiles acquisition.
Start position mode	Here you can define the alignment of the tile scan in respect of the defined position.
- Center	If selected, the start position is the center of the tile scan.
- Upper Left	If selected, the start position is the upper left corner of the tile scan.
Navigation	Click on the arrow button to show the section in full. Here you can check if each single image of your tile image is in focus before you start the acquisition. Navigate from on tile to another by using the arrow buttons or enter the X/Y value, to check the set-up of your z-stack.
Start position	Displays the X and Y value of the starting position for the tiles acquisition.

12.5.3.6 Channel

With this tool you control and adjust the laser. The following parameters are available:

Parameter	Description
405 nm	Here you can set the required attenuation (in %) of the laser using the slider, the arrows, or typing in the input field.
Pinhole	Adjusts the diameter of the pinhole. The diameter is specified in micrometer. The text below translates this diameter to Airy Units (AU) and section thickness. For confocal topography measurements a pinhole size of 1 AU is recommended.
- 1 AU	Sets the pinhole diameter to the recommended value of 1 AU (Airy Unit).
- Max (Default)	Opens the pinhole to its maximum diameter. This is also the default setting for the pinhole. This allows you to easily focus on the surface just like with a widefield microscope. Of cause focusing via the eyepieces is also possible.
Master Gain	Here you can control the voltage of the PMTs. Higher voltage increases the gain of the PMT. The image becomes brighter and you may be able to reduce the laser power. At higher voltage, the noise level in the image increases. The optimum between gain and noise depends on your experimental requirements and on your sample. The maximum available voltage for multialkali PMTs is 900V.

Parameter	Description
Digital Offset	Here you can perform adjustments on the background of the image.
Digital Gain	Here you can digitally amplify the laser signal.

12.5.3.7 Acquisition Mode

Here you adjust scanning and acquisition parameters that you want to apply for the entire experiment.

Parameter	Description
Frame Size	Adjust the frame size (in pixel) of the displayed image by entering the desired value in the two input fields. To change the frame size you must stop the live image acquisition.
- **Presets** button	By clicking on this button you can select from a list of default frame sizes (e.g. 128 x 128 or 512 x 512). In case of topography and layer thickness measurements we recommend to start with 1024 x 1024.
Scan Speed	Set the scan speed by adjusting the slider from 1 (slow) to 16 (very fast). Note that the available maximum scan speed depends on the selected **Frame Size** and zoom factor.
- **Confocal** button	By clicking on this button the frame size (image resolution) will be set to an optimal value corresponding to the optical magnification (objective) and the zoom factor . This provides an image where no information is lost and no empty data are generated as optimal sampling is achieved. The optimal value is calculated for the given objective and magnification settings matching a 2fold oversampling according to 2 fold Nyquist. Rectangular image dimensions are preserved.
Direction	Following scanning directions can be selected:
- Unidirectional	The laser scans in one direction only, then moves back to scan the next line.
- Bi-directional	The laser also scans when moving backwards, i.e. the scan time is halved. In case a pixel shift between forward and backward movement (double image), resulting from bidirectional scanning, is visible, use the **Correction X** / **Correction Y** sliders to correct it. By clicking on the **Auto** button an automatic scan correction will be performed.
Averaging	
- Number	Select the number of images you want to average (1 - 16).

Parameter	Description
- Method	Select the method which will be used for averaging: ▪ **Mean Intensity**: Uses the mean average of all images ▪ **Sum Intensity**: Uses the sum of all images.
- Bits per Pixel	In the dropdown list you can adjust the color bit depth to 8 Bit or 16 Bit (i.e. 256 or 65536 gray values). To change the bit depth you must stop the live image acquisition.
Scan Area	In this section, you can adjust the position of the scan area. The outer frame corresponds to the field of view of the microscope. The inner frame represents the scan area. All changes (Offset, Rotation, Zoom) made in this section will be immediately applied to the scan area. Following functions are available:
- Offset	Adjust the offset by using the **Left** / **Right** or **Up** / **Down** sliders. You can also enter a specific value in the input field. If clicking on the **C** button behind the input filed the offset position will be reset to center position. If you left click on the inner frame and hold down the mouse button you can move the scan area freely. The positions in the input fields will be adopted according to your adjustments.
- Rotation	Adjust the rotation degree by using the **Rotation** slider. You can also enter a specific value in the input field. If clicking on the **O** button behind the input field the rotation degree will be reset to default position (zero degree).
- Zoom	Adjust the zoom level (from **0.5x** - **40x**) by using the **Zoom** slider. You can also enter a specific value in the input field. If you click on the **1/2** button behind the input field the zoom level will be reset to default (0.5x).
- Reset Scan Area	Resets all adjustment to the system defaults.

12.5.3.8 Z-Stack

Here you setup the acquisition of the Z-Stack image.

Info

Z-stack images are always acquired from bottom to top automatically, irrespective of whether you have defined the top or bottom Z-plane of your stack as the first Z-plane. This acquisition sequence increases the accuracy of the Z-positioning.

For manually configuring Z-Stacks you have two modes available:

Parameter	Description
First / Last	If activated, you are able to configure the Z-stack via setting the first and the last positions of the Z-stack, see *Configuring a Z-Stack manually (First/Last Mode) [48]*.
Center	If activated, you are able to configure the Z-stack via setting the center plane of the Z-stack, see *Configuring a Z-Stack manually (Center Mode) [48]*.

Depending on which mode you have activated, you will see the following parameters for configuring the Z-stack:

Parameter	Description
Set Last/Set First	Only visible for **First/Last** mode. By clicking on the **Set Last** and on the **Set First** button you determine the current position as last or first position of the Z-stack.
Range	Displays the range of the configured Z-stack from the last to the first section plane.
Slices	Here you can enter the number of Z-slices that the Z-stack will have.
Interval	Here you can enter the desired distance between the Z-slices.
Optimal	The number on this button shows the distance calculated for the channels set and the current microscope according to the Nyquist criterion. If you click on the button, this value is automatically adopted into the **Interval** input field.
Keep	▪ **Interval**: Keeps the set interval between the section planes constant if you change configuration parameters in the Z-Stack tool. ▪ **Slice**: Keeps the set number of Z-slices constant.
Center	Only visible for **Center** mode. If clicking on this button the current position is set for the central Z-plane. You can also enter the value in the input field to the right of the button.
Offset	Here you can enter a value for an offset if desired.

12.6 Correlative Array Tomography (CAT)

This module enables you to perform automated imaging of ultra-thin serial sections (ribbons) using the light- and scanning electron microscope. After calibration of the sample carrier and detection of the sections, regions of interest can be defined manually in a single section that will be automatically propagated to all sections. The selected regions of interest can then be imaged with different contrast methods and magnifications using the LM.

In the SEM the previously defined regions of interest will then be imaged automatically after loading the image previously acquired at the LM. The corresponding 2D image sequences recorded by the LM and SEM are aligned into a 3D Z-Stack using the integrated alignment and correlation algorithms of the ZEN Correlative Array Tomography module. This process results in a correlative 3D data set combining LM and SEM information into one image volume.

For the correlative workflow, one CAT module has to be installed on the widefield system, a second module hast to be installed on the SEM. A detailed how-to guide of the workflow can be found in the chapter *The CAT Workflow [▸ 273]*.

The software module can be used with ZEISS widefield microscopes as well as with ZEISS scanning electron microscopes. In addition to the CAT tool the module offers four wizards. Detailed descriptions of the functions of the tools and wizards can be found in the linked chapters.

- **Correlative Array Tomography** tool, see *CAT Tool [▸ 303]*.
- **Calibration Wizard**, see *Sample Holder Calibration Wizard [▸ 304]*.
- **Acquisition Wizard**, see *Acquisition Wizard [▸ 307]*.
- **Z-Stack Alignment Wizard**, see *Z-Stack Alignment Wizard [▸ 314]*.
- **Correlation Wizard**, see *Correlation Wizard [▸ 316]*.

12.6.1 Basics of Array Tomography

Array Tomography is a volumetric microscopy method employed to visualize and reconstruct 3D images of serial sections. Tissue samples or cells embedded in resin are cut into consecutive sections with an ultramicrotome and collected onto a sample carrier (e.g. cover glass). The sequence of the sections determines the z-position and allows the reconstruction of the 3rd dimension. Therefore the z-resolution of the resulting 3D data set is determined by the thickness of the section.

The correlation of scanning electron microscope (SEM) data especially with an image acquired using a fluorescence light microscope (LM), enables the visualization of fluorescently labeled biological structures in their ultrastructural context not only in 2D but now in 3D with the **ZEN Correlative Array Tomography** module.

12.6.2 Sample Preparation

Type of Sample Carrier / Cover Glasses

We recommend cover glasses coated with Indium tin oxide and fiducials. Indium tin oxide minimizes charging effects in the scanning electron microscope. Cover glasses with fiducials enables additional preparation steps after imaging the sample with the light microscopes and before imaging with a scanning electron microscope.

Deposition of Serial Sections on the Cover Glass

- **Sequence of serial sections**
  During serial sectioning, make sure that you know the sequence of the ribbons as well as the start and the end point of the ribbons.
- **Positioning of serial sections on a cover glass**
  It is important to position the serial sections in the center of the cover glass. If the sections are too close to the edge of the cover glass, it might happen that the objective touches the sample carrier during the image acquisition. This might happen particularly for immersion objectives. The consequence will be that the focus map is not calculated in the correct way or images are out of focus.
- **More than one ribbon (serial section) on a cover glass**
  Take care that the single serial sections are not in close contact to each other, this might confuse the numbering algorithm of the software and creates a wrong numbering.

12.6.3 Pre-Settings (Light Microscope)

Before you can start working with the CAT module, you have to check the following settings on the light microscope system (hardware and software settings). In general the system is calibrated by a service technician but we recommend to check the settings again especially when you have changed components e.g. objectives or filter cubes. As these general settings are not described here in detail please ask your service technician or read the ZEN Online Help.

- **Check Parcentricity and Parfocality**
  Note that the calibration of parcentricity and parfocality has to be done at the TFT display of the microscope.
- **Check Camera Orientation and Stage Movement**
  Before checking the stage movement the correct camera orientation always has to be set first. The camera orientation should match your view through the ocular of the microscope. If this is not the case you can change the camera orientation in the **Camera** tool.
  To check the stage movement we recommend to acquire a tiles image and check if the tiles are put together correctly. If this is not the case you have to close ZEN software and open the **MTB 2011** software. There you must change the stage inversion in the configuration list under **Motorized Stage**.
- **Perform Shading Correction**
  Before starting the CAT workflow a shading correction has to be performed in ZEN software. Please read the corresponding chapter in the ZEN Online Help.

12.6.4 Experiment Settings

For working with the CAT module you need to set up an experiment in the ZEN software first. As this is already described in the ZEN Online Help, we will focus here on the most important settings which are essential for the CAT workflow:

- In the menu **Tools** | **Options** | **Acquisition** | **Acquisition Tab** the checkbox **Enable Advanced Imaging Setup** must be activated.
- In the **Imaging Setup** tool **Show All** must be activated.
- In the **Imaging Setup** tool **Advanced Imaging Setup** must be selected.
- In the **Imaging Setup** tool in the lightpath display the following settings have to be adjusted:
  - The **Microscope Manager** must be excluded from all settings. To exclude a setting, click on the icon of the component / setting. You will see a menu where you can activate/deactivate the checkbox **Include in this Setting**. If the checkbox is not activated, the component/setting is excluded from the selected setting. Note that you have to select each Before/After setting and check if the components are excluded or included and adjust it accordingly.

- The **TL / RL Switch** must be excluded from all settings.
- All **TL / RL Shutters** in the light path must be included in all settings. Note that icons of included settings are highlighted in blue color.
- All **light sources** (TL / RL) within the light path must be included in all settings.
- The light intensity of the light sources for all settings must be the same (e.g. 2V).

- In general for the **Before Experiment** and **After Experiment** settings adjust the following:
  - All **Shutters** must be closed.
- Example for Phase Contrast settings:
  - For the **Before TL Phase** settings adjust the following:
    - The **TL Shutter** must be opened
    - The **RL Shutter** must be closed
  - For the **After TL Phase** adjust the following:
    - The **TL Shutter** must be closed
    - The **RL Shutter** must be closed
- Example for one Fluorescence Channel (here DAPI) settings:
  - For the **Before DAPI** settings adjust the following:
    - The **TL Shutter** must be closed.
    - The **RL Shutter** must be opened
  - For the **After DAPI** adjust the following:
    - The **TL Shutter** must be closed.
    - The **RL Shutter** must be closed

12.6.5 The CAT Workflow

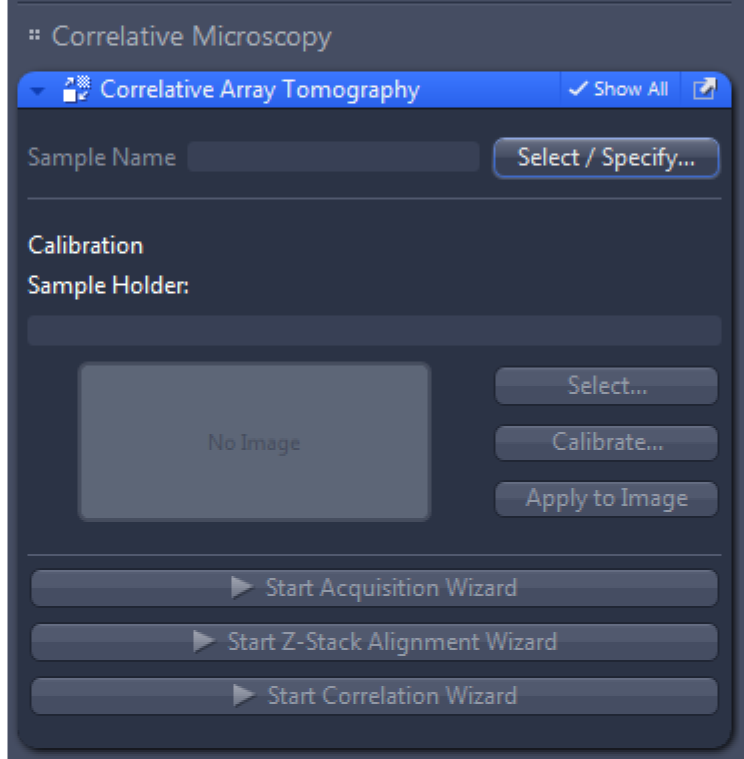
In this chapter you will find how-to guides describing the typical CAT workflow. The chapter is for users who search for an introduction to the CAT module and workflow. Starting from general preparations to the acquisition on the LM (light microscope), we will also explain how to acquire images with the SEM (Scanning Electron Microscope). After the image acquisition we will focus on the image alignment and correlation.

Please note that we will not explain how to set up an experiment in detail as this step is beyond the scope of this guide which is focused mainly on the CAT workflow. Instead of that please read the chapter *General Preparations [▸ 273]*, where we describe the most important pre-requisites for a CAT experiment. We will not take a look at the further processing of the resulting images as well.

12.6.5.1 Creating a new sample

If you have configured your experiment in ZEN (e.g. a multi-channel experiment) the next step is to create and select a sample. When you work with the software for the first time you have to create a new sample first.

Prerequisite ✓ You are in the **Correlative Array Tomography** tool.



1. Click on **Select** / **Specify**.
   → The **Select Sample** dialog opens.
2. Click on the [+] **Add** button under the **List of specified samples**.
   → The **New Sample** dialog opens.
3. Enter the necessary sample information: Name, Description, Number of sample carriers, Type of sample carrier and Section thickness.
4. Click on **OK**.
   → The dialog closes. You will see the new sample in the **List of specified samples**.
5. Select the new sample from the list and click on **OK**.

You have created and selected a new sample.

Info

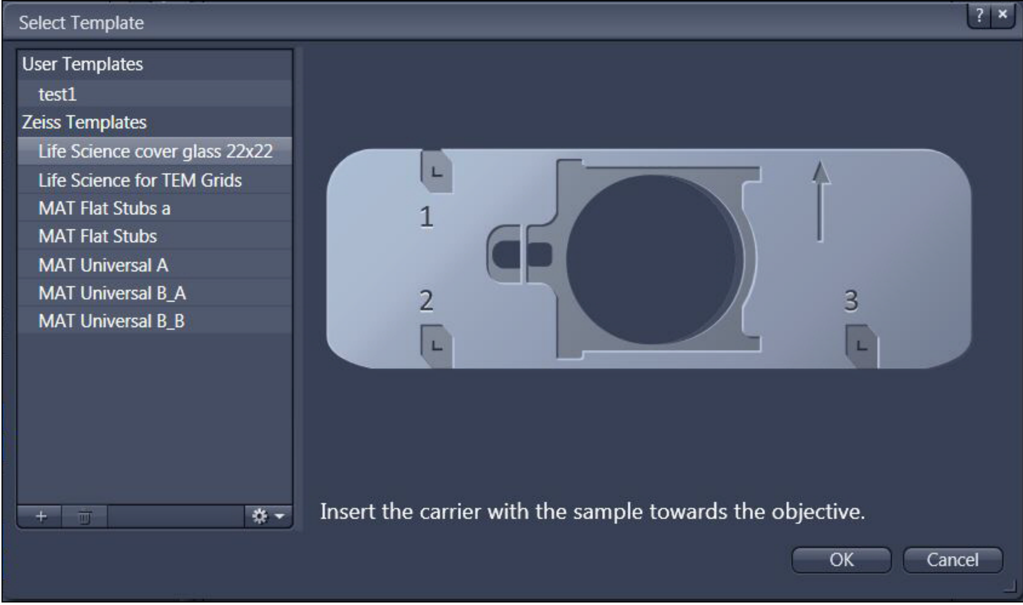
Note that specifying the correct number of sample carriers is important for the numbering of the ribbons/sections afterwards. The sample information will be stored within the image data and will be used for further image processing and data management.

12.6.5.2 Selecting the Sample Holder

Prerequisite ✓ You are in the **CAT** tool.

1. In the **Sample Holder** section click on **Select…** to open the **Select Template** dialog and to choose the correlative sample holder you want to use. Different types of correlative holders are available, see Appendix *Correlative Sample Holders [▸ 318]*.

2. In the **Select Template** dialog select the correlative holder you want to use. If you want to use your own sample holders, click on the **Add** button below the list and follow the instructions in the chapter *Defining new sample holder templates* [277].



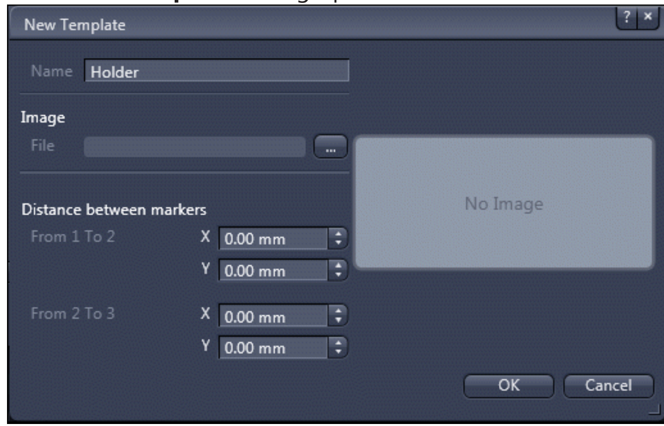
3. Click on **OK** to close the dialog.

You can now continue with the calibration of the sample holder, as described in the chapter *Calibrating the sample holder* [278]. Note that the calibration of the sample holder is mandatory to acquire images.

12.6.5.3 Defining a New Sample Holder Template

With this dialog you can define new correlative holders in addition to the existing holder templates. It is not mandatory to use correlative holders from ZEISS. User-defined correlative holders with 3 fiducial markers can be used as well.

1. To open the dialog click on **Add** in the **Select Template** dialog. This dialog can be opened via the **Shuttle & Find** tool.
   → The **New Template** dialog opens.



2. Type in a name for the new holder / sample carrier. An image of the new holder can be loaded as well.
3. Insert the distances (in millimeters) between the first and the second marker and between the second and third marker.

→ The distances can be determined using the **Stage Control** dialog accessible via the **Light Path** tool in **Right Tool Area** tab. We recommend to do this before you start the New template dialog. Write down the distances to be prepared to enter them within the New Template dialog.

→ Activate the live view in the Center Screen Area by clicking on the **Live** button in the Locate tab.

→ Navigate the stage manually to the calibration marker on the sample holder by means of the joystick and note the x/y-coordinates of the marker.

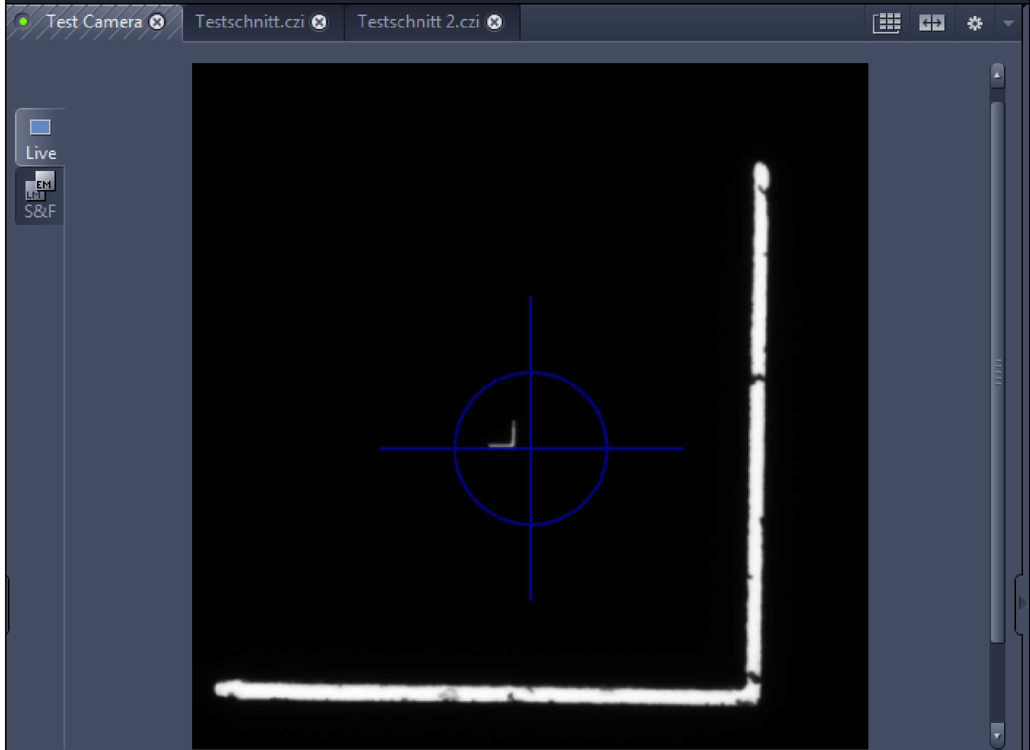
→ Repeat this procedure for all three markers and calculate the distances between marker 1 and marker 2 and between marker 2 and marker 3, respectively.

12.6.5.4 Calibrating the Sample Holder

Correlative sample holders have three fiducial markers enabling a three point calibration (signed with the numbers 1-2-3) The calibration markers consist of one small (length 50 µm) and a large L-shape marker (length 1 mm). The bigger marker is used for coarse orientation, whereas the smaller marker is used for the calibration.

12.6.5.4.1 Preparing Calibration

1. Click on **Live** in the **Acquisition** tab to activate the live view in the **Center Screen Area**.
2. Navigate the stage manually to the first calibration marker on the sample holder (marked with No. 1) by means of the joystick. It is enough if you move the stage to the larger L-shaped calibration marker. The smaller marker will be detected automatically within the **Sample Holder Calibration Wizard**.To locate the marker positions we recommend to use a dry objective with low magnification (5x – 20x).



3. Open the **CAT** tool.
4. Click on **Calibrate…** to open the **Sample Holder Calibration Wizard.**

12.6.5.4.2 Setting Calibration Options

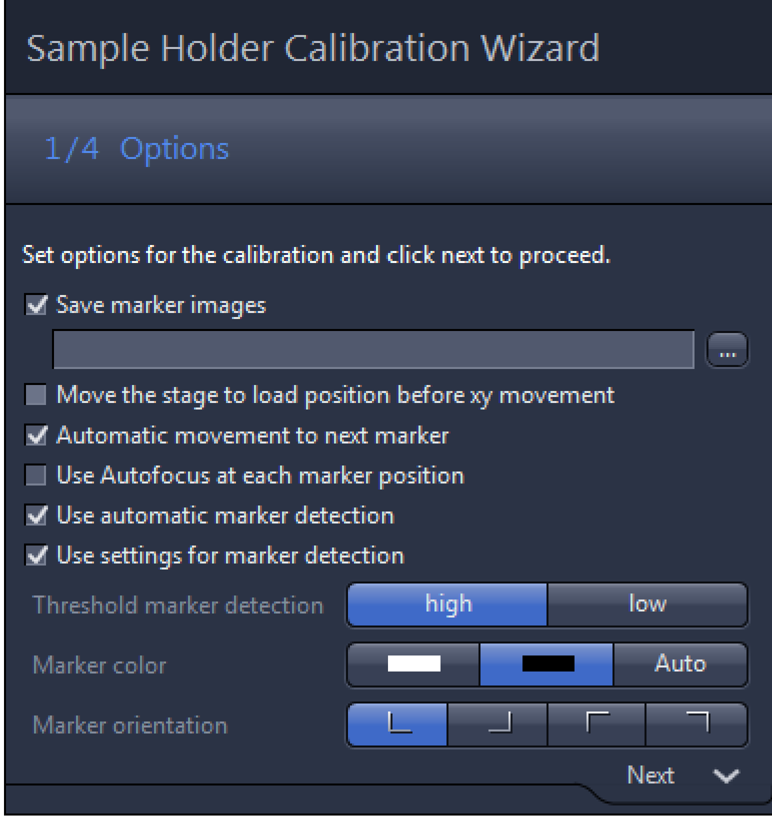


Fig. 13: Sample Holder Calibration Wizard Options

In step 1 of the wizard, the following options should be activated to follow our recommended workflow:

1. Check if the **Automatic movement to next marker** checkbox is activated.
   → This will automatically move the stage to the next marker position after you have confirmed the position of the marker and clicked on **Next**.
2. Check if the **Use automatic marker detection** checkbox is activated.
   → The software will try to find the correct positions of each marker automatically.
3. If you need to change the marker color, or check if the marker orientation is set correctly, activate the **Use settings for marker detection** checkbox to access these functions.
4. Click on **Next** to move to the next wizard step.

12.6.5.4.3 Performing Calibration

1. Click on **Set** to detect the first marker position.
   → An automatic stage calibration will be performed. After the stage calibration, the system will try to detect the marker position of the small marker automatically.
   → A message appears which asks if the marker was detected correctly.
2. Click on **Yes** to confirm the message.
3. Click on **Next** to move to the next step of the wizard.
   → The stage will automatically move to the next (coarse) marker position. If the stage moves into the wrong direction you can use the **invert X** / **invert Y** buttons to correct the movement direction.
4. Repeat the previous steps and set marker position 2 and 3 accordingly.

→ After setting marker position 3 you will find a green check mark icon which shows that the calibration was successful.

5. Click on **Finish** to save the calibration and close the wizard.

To check if the calibration was successful acquire an image and open the **Tree** view in the Center Screen Area. There you should see the correlative calibration data in the list. If the Tree view is not visible go to **Tools** | **Options** | **Documents** and activate the **Enable Tree View** checkbox.

12.6.5.5 Acquiring the LM image

The image acquisition will be performed by the help of the **Acquisition Wizard** which is opened if you click on **Start Acquisition Wizard** in the **Correlative Array Tomography** tool.
The wizard contains the following 7 steps:

- *Overview Imaging [▸ 280]*
- *Ribbon Definition (optional) [▸ 281]*
- *Ribbon Imaging (optional) [▸ 282]*
- *Section Specification [▸ 284]*
- *ROI Specification [▸ 288]*
- *ROI Imaging [▸ 289]*
- *Re-Shoot (optional) [▸ 290]*

12.6.5.5.1 Acquiring the overview image

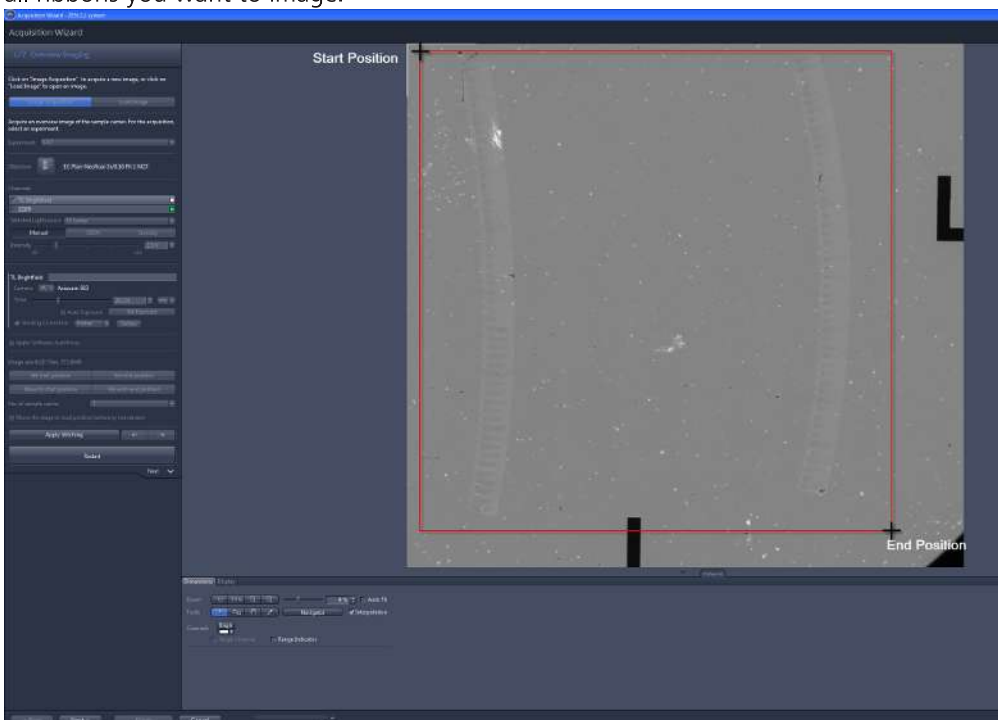
Info

Take care that the **Auto** checkbox on the **Dimensions** tab is deactivated.

Prerequisite
- ✓ You have started the **Acquisition Wizard** via the **CAT** tool.
- ✓ You are in step **1/7 Overview Imaging**.

1. Check if **Image Acquisition** mode is selected. This is the default setting when entering the wizard.
2. From the **Experiment** dropdown list select the experiment that you have prepared in advance.

3. From the **Objective** list select an objective with a low magnification, e.g. 5x.
4. Select the **Channel** and the **Light Source** you want to use for acquiring the overview image. For the overview image we recommend to select **Phase contrast** as channel mode.
5. Move the stage to the upper left corner of your sample.
6. Click on **Set start position** to define the starting position of the overview image.
7. Move the stage to the bottom right corner of your sample.
8. Click on **Set end position** to define the end position of the overview image.
9. Click on **Acquire Overview Image**.
   → The overview image will be acquired. Then you should see the complete sample showing all ribbons you want to image.



10. Click on **Apply Stitching** to remove the offset between the single tile images.

You have successfully acquired the overview image. You can now continue with the next step by clicking on **Next**.

12.6.5.5.2 Defining the ribbons

Info

When no detailed sample information is necessary to identify regions of interest within the sections, you can skip this step and the following step 3 Ribbon imaging as well. You can then go on with the wizard step 4 *Section specification* [▸ 284].

Prerequisite ✓ You are in step **2/7 Ribbon Definition** of the Acquisition Wizard.

1. Use the tools on the **Ribbon Definition** tab to mark the contour lines of the ribbons which should be imaged. The contour lines are displayed in yellow color.

→ The software will automatically create as many tiles as necessary for imaging the ribbons. The number of the tiles depend on the selected objective. The frames of the tiles will be displayed in red color.

2. When you have marked the contours, click on **Next**.

See also

Specifying the sections [▸ 284]

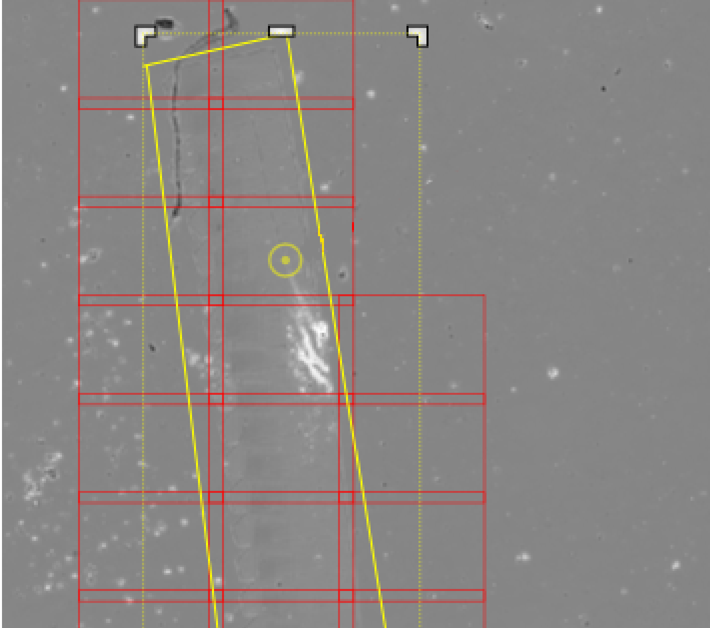
12.6.5.5.3 Imaging the ribbons

Please note that this is an optional step and must be performed only when you have defined ribbons as described in step 2. In summary, you have to perform the same actions mentioned in step 1 but you should use an objective with higher magnification and apply the **Global** focus strategy under **Focus Surface**.

Prerequisite ✓ You are in step **3/7 Ribbon Imaging**.

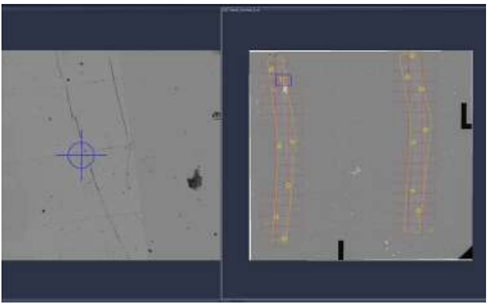
1. Under **Objective** select an objective with a higher magnification than for the overview image, e.g. 10x. The objective should enable you to clearly recognize the structures of interest on your sample.
2. Under **Focus Surface** select **Global (all Regions/ Ribbons)**.
3. Click on **Distribute Support Points...**.

→ The support points will be distributed automatically over the ribbons. They are displayed as yellow circles with a point in the middle.



4. You can add further support points if necessary by using the **Add** button [+] below the **Distribute Support Points** button. Simply click on the image at the position where you would like to add another support point.
5. Click on **Verify Support Points**.

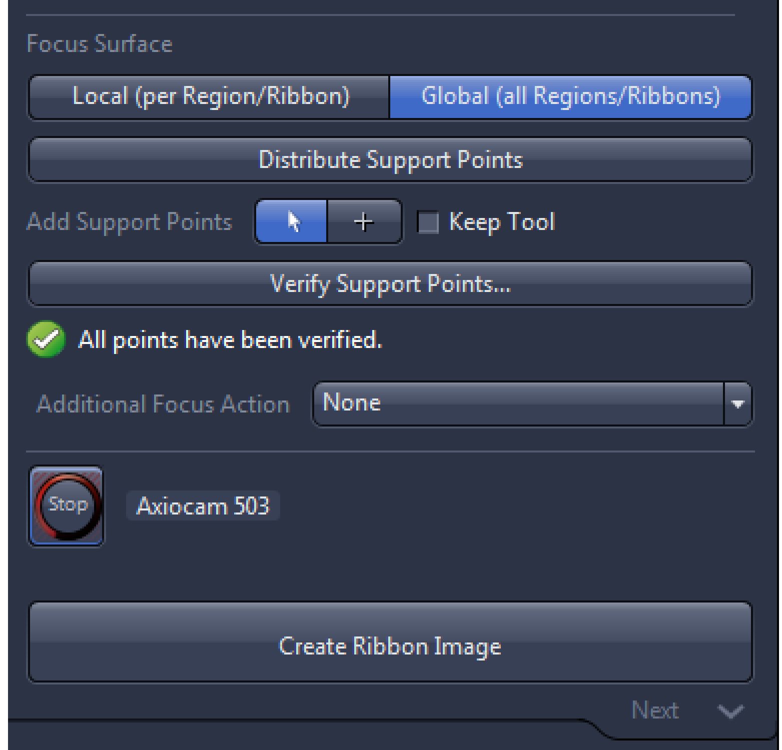
   → Now you can check if each support point is in focus. You will see the overview image in the right image container and the detail image in the left image container. The verification process will start with the first support point which was set. The current support point is marked with a red crosshair. When you activate **Show stage position within the image** on the **Ribbon Definition** tab below the **Center Screen Area** you will see the current position of the stage in the image as a rectangle with a blue dashed frame.



6. Hold *CTRL* key and use the mouse wheel to adjust the focus for the corresponding support point.
7. When the support point is in focus click on **Confirm**.

   → The software will automatically move to the next support point.

8. Repeat the last two steps until you have corrected and verified all support points. At the end of the process you will see the message **All points have been verified**.



9. Click on **Create Ribbon Image**.
   → The ribbon image will be generated. Note that each ribbon will be displayed as one scene.
10. Again we recommend to click on **Apply Stitching** to remove the offset between the single tile images.

You have successfully acquired the ribbon image and can now continue to the next step by clicking on **Next**.

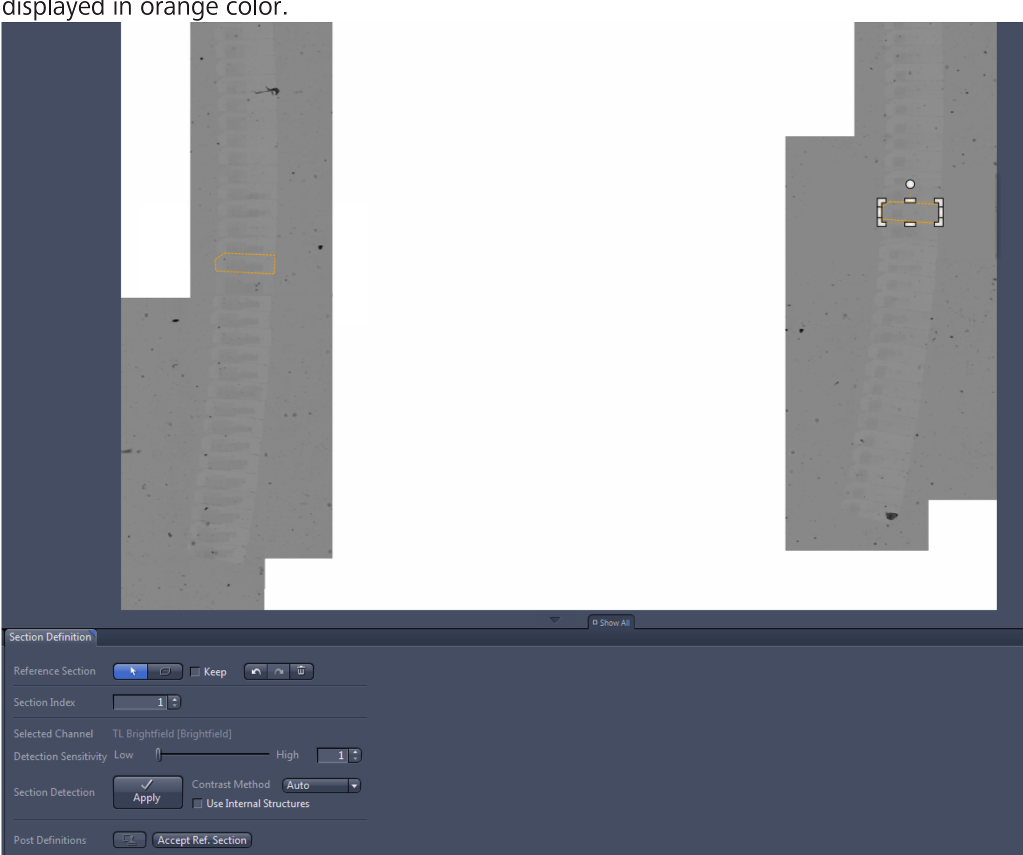
12.6.5.5.4 Specifying the sections

In this step all sections will be identified by using a section detection algorithm. In summary you have to mark the outline of at least one section on each ribbon. Then the section detection algorithm will detect the sections of the ribbon automatically. If the automatic section detection does not work properly or if not all sections are detected you can stamp in the missing sections. It is also possible to edit the shape, location and orientation of the section frames afterwards.

Prerequisite ✓ You are in step **4/7 Section Specification**.

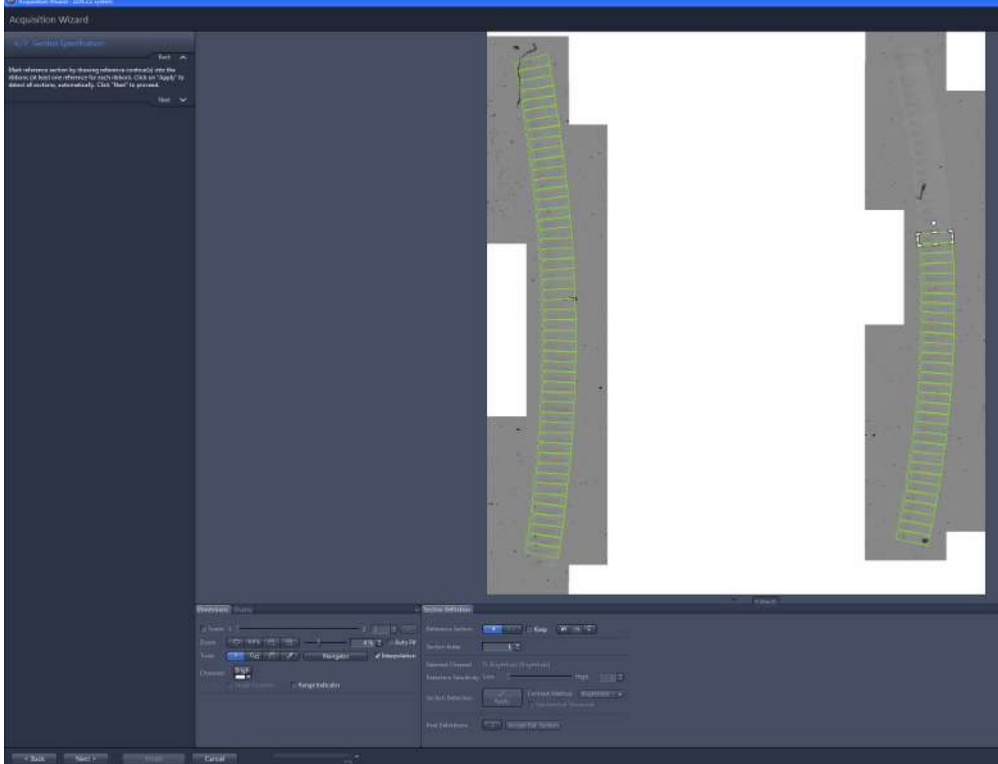
1. On the **Section Definition** tab select the **Polygon** tool.

2. Mark the outline of one section in each ribbon. The outlines of these reference contours are displayed in orange color.

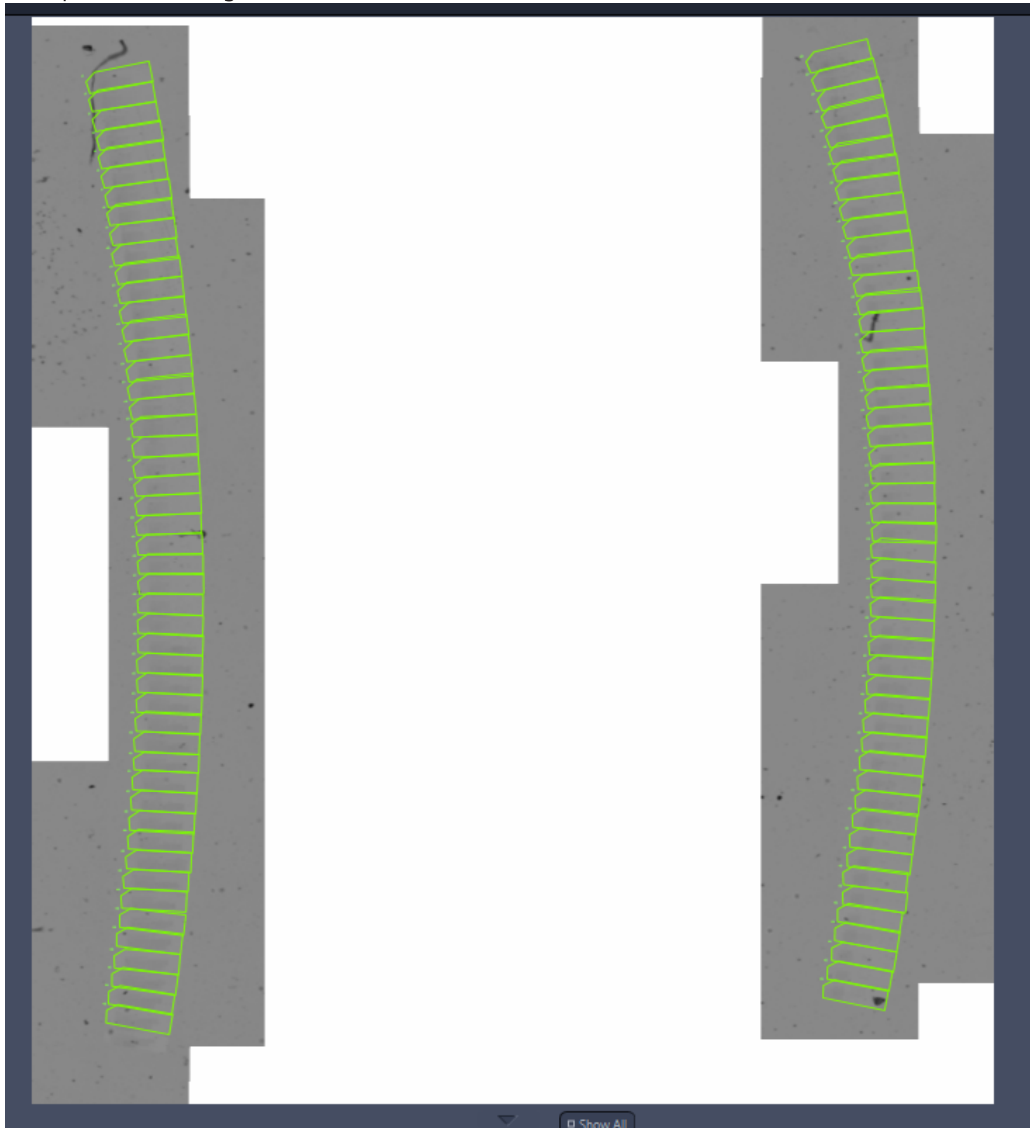


3. Click on **Apply**.

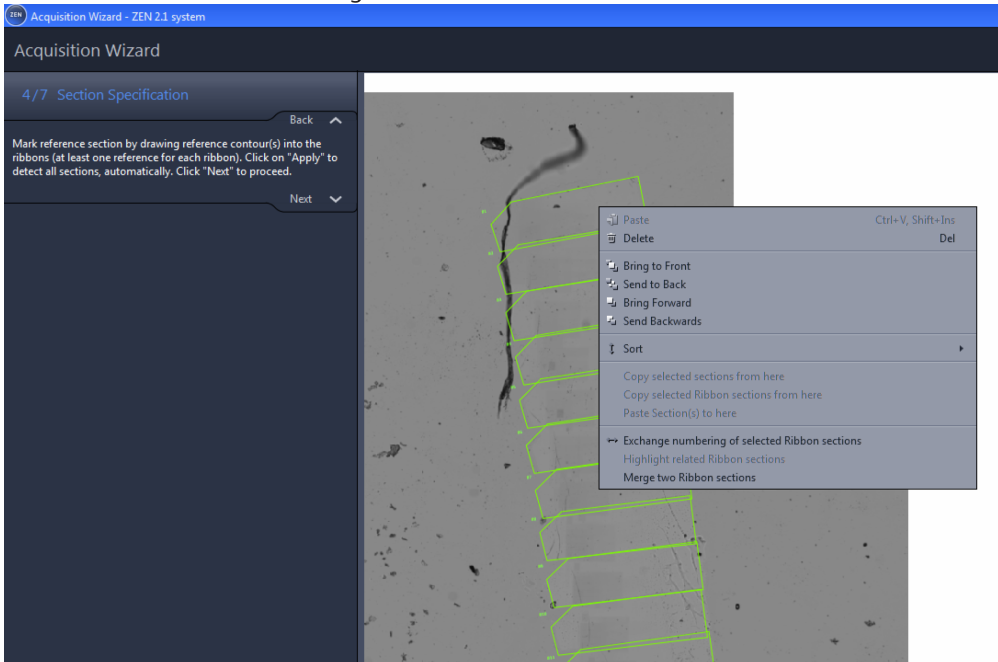
→ The software will try to detect the remaining sections automatically. When finished the detected sections appear in green color.



4. If not all sections can be detected, mark the last section which was detected and click on the **Stamp** tool in the **Section Definition** tab.
5. Stamp in the missing sections so that each section is marked.



→ Please take your time to check the numbering carefully. A correct numbering is prerequisite for a successful alignment of the sections, afterwards. The numbering of the ribbons depends on how you deposited the ribbons during the cutting. To adjust the numbering you have several options available in the context menu. To open the context menu move the cursor over a section and right click with the mouse.

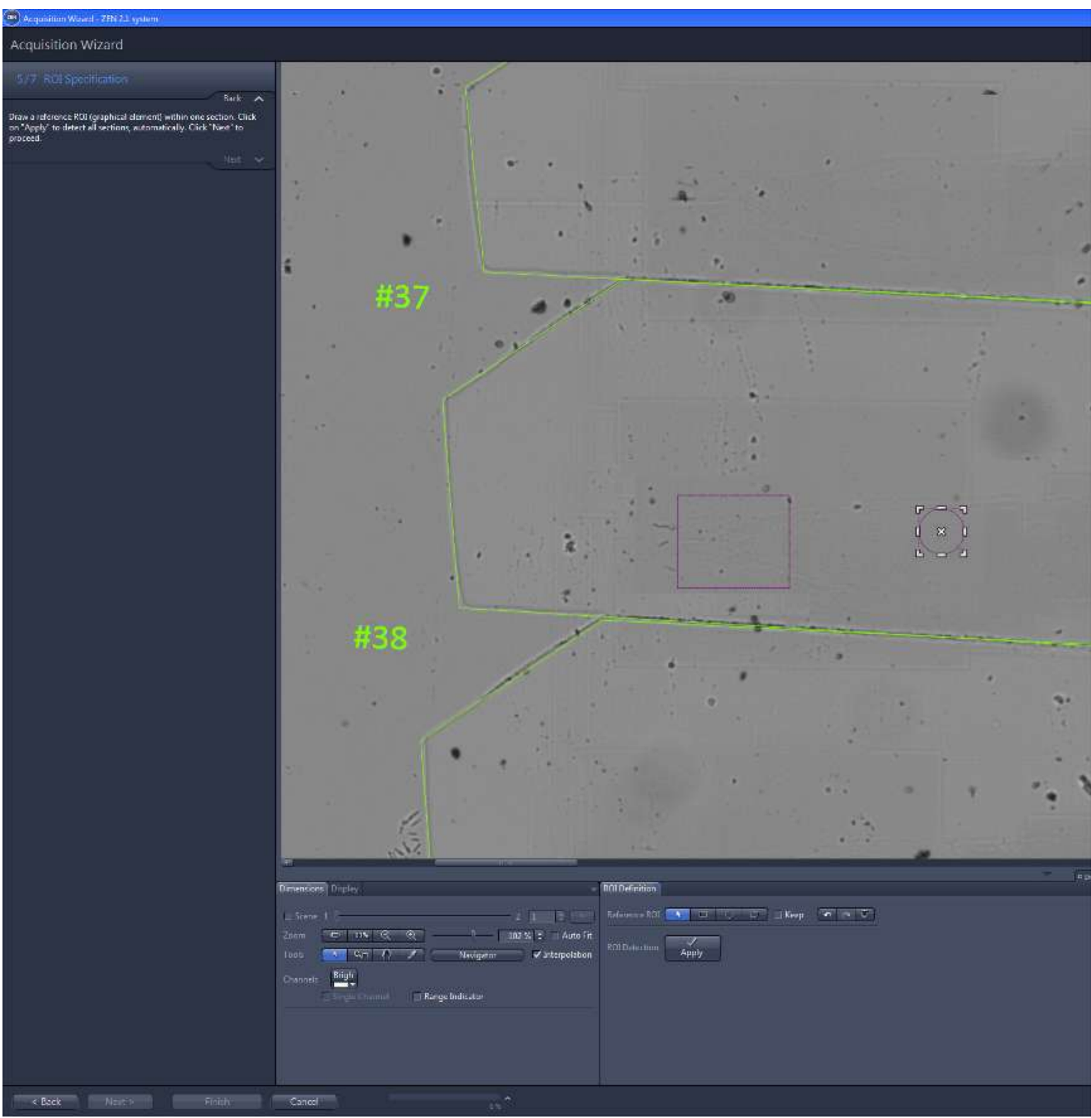


6. If the numbering is correct, proceed to the next wizard step by clicking on **Next**.

12.6.5.5.5 Specifying the ROIs

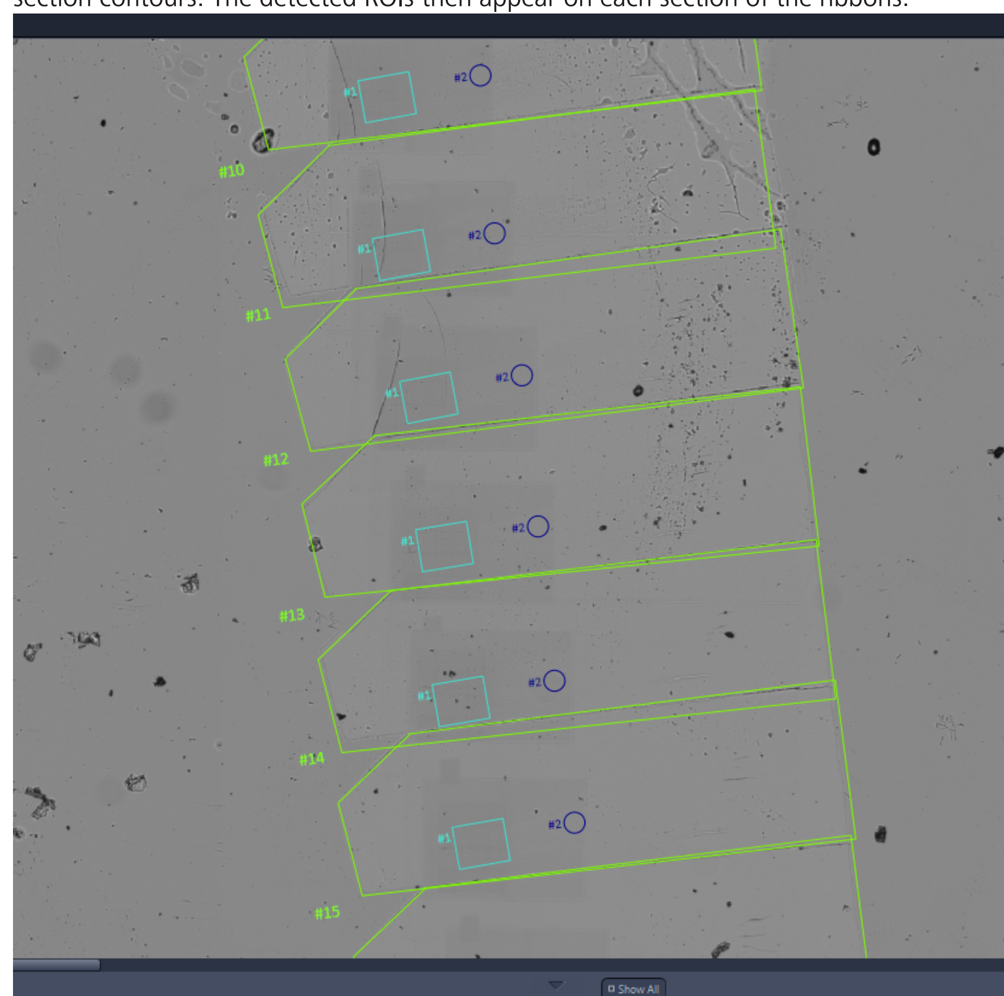
Prerequisite ✓ You are in step **5/7 ROI Specification**.

1. On the **ROI Definition** tab select the desired tool for marking a ROI, e.g. **Rectangle** or **Circle**.
2. Mark the desired ROIs in one section. Marked ROIs will be displayed in purple color.



3. Click on **Apply**.

→ The software will position the defined region of interests in each section according to section contours. The detected ROIs then appear on each section of the ribbons.



If the ROIs are detected correctly proceed with the next wizard step by clicking on **Next.**

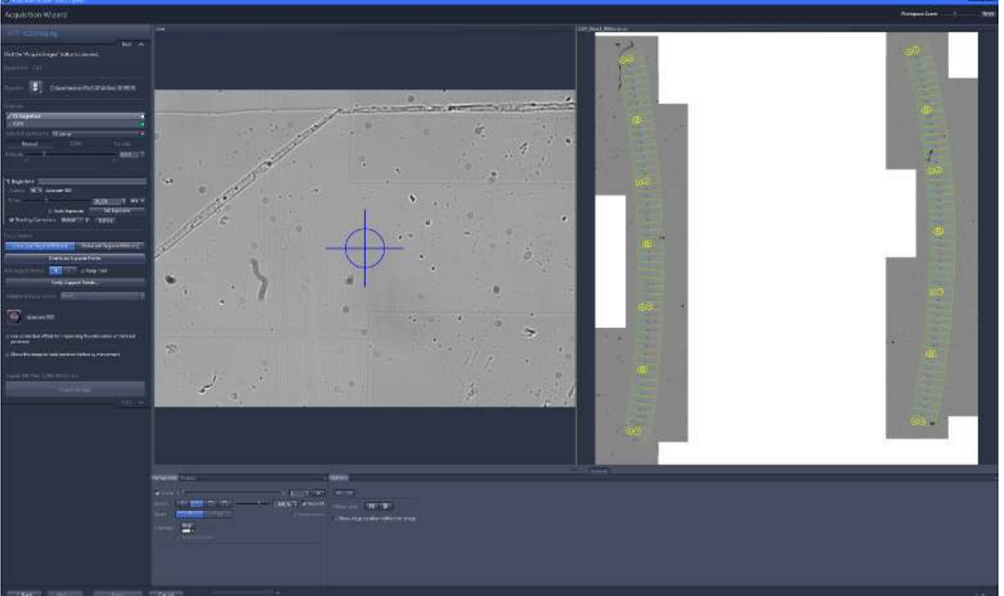
12.6.5.5.6 Imaging the ROIs

In this step we will image the ROIs using a high magnification objective and apply a local focus strategy. This will result in very detailed and sharp images of the ROIs which are used for the further processing (e.g. creating Z-Stacks and image correlation with SEM images).

Prerequisite ✓ You are in step **6/7 ROI Imaging**.

1. Under **Objective** select an objective with a high magnification, e.g. 63x.
2. If you are using fluorescence samples you can now activate the corresponding fluorescence channel.
3. Under **Focus Surface** select **Local (per Region / Ribbon)**.
4. Click on **Distribute Support Points**.

→ According to the step **3 Ribbon Imaging** the support points will be distributed automatically. The support points are distributed alternately outside and inside a ROI to guarantee best focusing results.



5. Click on **Verify Support Points**.
   → The software will guide you through the process in the same way you were been guided in step **3 Ribbon Imaging**.
   → If you have verified all existing support points the message **All points have been verified** will appear.
6. Click on **Acquire Images**.

The ROIs will be imaged now. You can use the next step to check each image of the ROIs and re-acquire (re-shoot) images from ROIs which do not fit your expectations. To proceed with the next step click on **Next**.

12.6.5.5.7 Re-Shooting ROIs

This step is basically used for re-acquiring images from ROIs that are out of focus.

Prerequisite ✓ You are in step **7/7 Reshoot**.

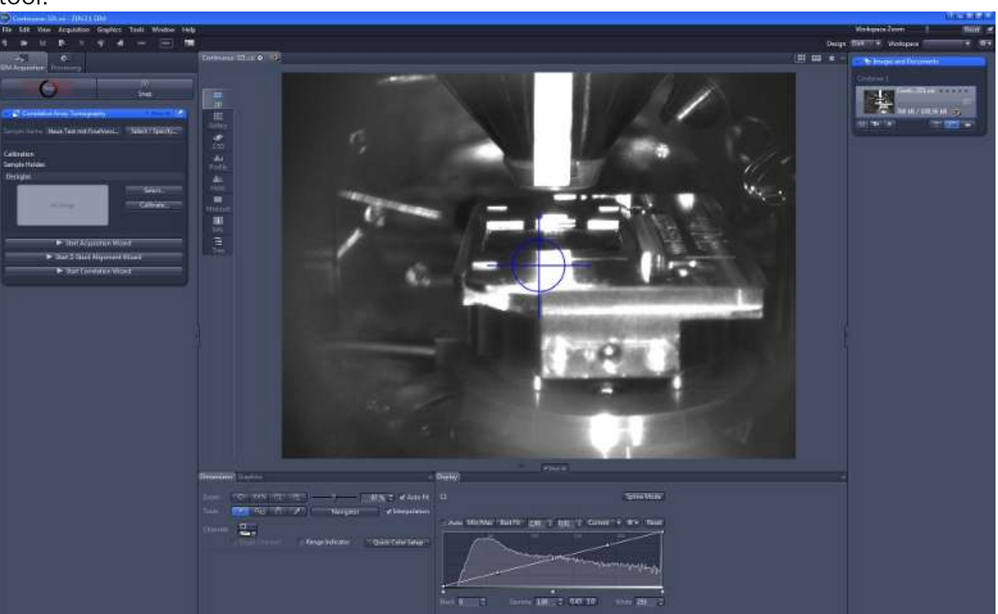
1. Check if the **Select Tiles** mode is activated. This mode should be selected by default when entering this step.
2. You can use the arrows on each side of the image to navigate through the ROI series or the **Z-Position** slider on **Dimensions** tab.
3. Select an image you want to re-acquire by clicking on it with the left mouse.
   → The image will be displayed with a dashed green frame.
4. Continue with checking the images and selecting the images you want to re-acquire.
5. When you have selected all images to re-acquire click on **Acquire**.
   → The software will move to the first image which you have selected.
6. Adjust the focus and click on **Snap**.
7. Click on **Replace** to replace the old image with the new one.
   → The software will automatically move to the next selected image to be re-acquired.
8. Continue until you have re-acquired all selected images.
9. Click on **Finish**.

You have successfully completed the Acquisition Wizard for the light microscope images of your ribbons. Continue with the process described in the next chapter of this guide.

12.6.5.6 Preparations for the SEM image

Prerequisite
- ✓ You have acquired the LM image according to the instructions in the chapter *Acquiring the LM image [▸ 280]*.
- ✓ You have copied/transferred the image data of the light microscope to the SEM PC.

1. Start the **Smart SEM** software. Note that the SEM software is used for setting up the acquisition parameters, e.g. detector settings, magnification, display settings and scan speed.
2. Start the **ZEN SEM** software. Please take care that before you start ZEN , SmartSEM was started.
   → You will see the **SEM Acquisition** tab and the **Correlative Array Tomography** (CAT) tool.



3. In the CAT tool click on **Select / Specify ...**.
4. Select the sample file which you have created during the LM acquisition. It should be located in the LM folder which was generated automatically.
5. Select the sample holder you are using for your sample.
6. Calibrate the sample holder like described in the chapter Calibration.

You have successfully finished the general preparations. You can now continue with the acquisition of the SEM image.

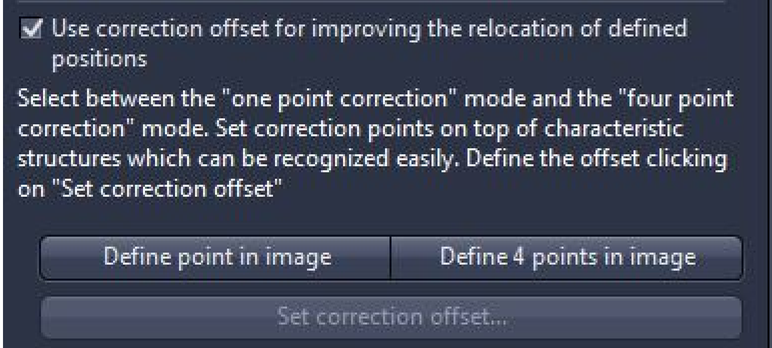
12.6.5.7 Acquiring the SEM image

Prerequisite
- ✓ You have done the general preparations, see *Preparations for the SEM image [▸ 291]*.

1. In the **CAT** tool click on **Start Acquisition Wizard**.
   → You will see the first step **Overview Imaging** of the wizard.
2. Click on **Load Image**.
3. Select the image file containing the ROIs from the CAT / LM folder on your file system.

→ You will see the image containing the ROIs.

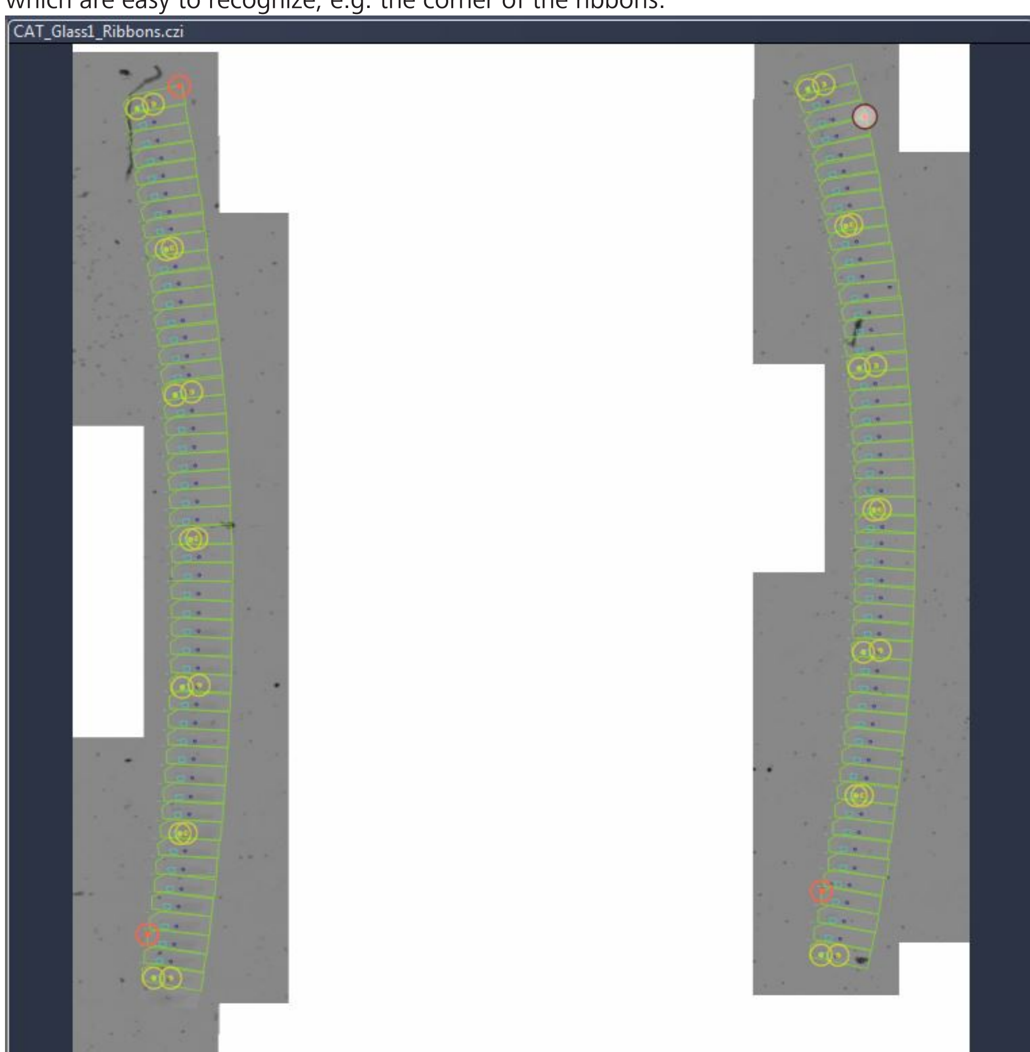
4. Click on **Next**.
   → The wizard will now jump directly to step **6/7 ROI Imaging** as the software recognizes the marked ROIs in the image file.
   → Before starting to acquire the ROIs we recommend to perform the offset correction. Therefore proceed as follows:
5. In the ROI image click on a position with a prominent structure.
   → The stage will move to that position automatically. Now you may recognize a difference between the position you have clicked on and the actual position. This is the offset we want to correct now.
6. Activate the offset correction checkbox.



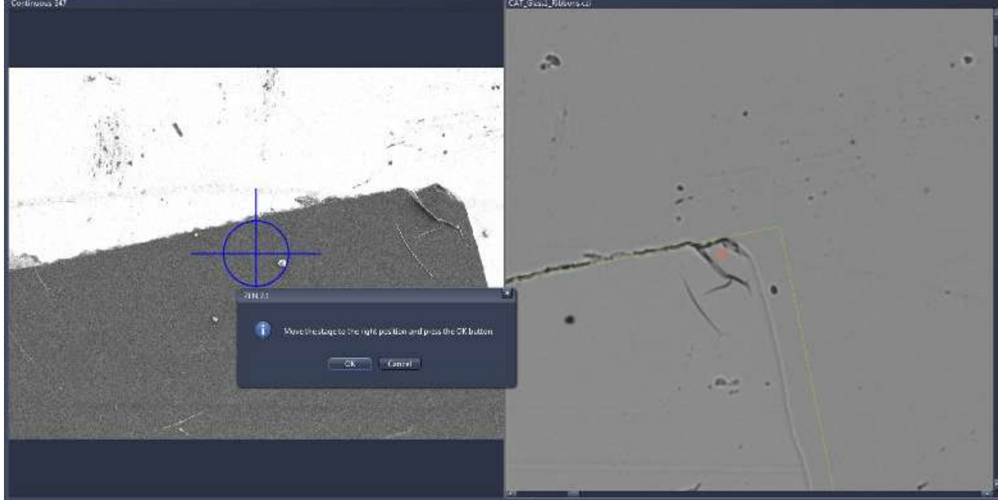
   → The controls for the offset correction will appear.
7. Click on **Define 4 points in image**.

→ The 4 correction points are distributed automatically within the image. The correction points look like the support points (red outline with a red dot in the middle).

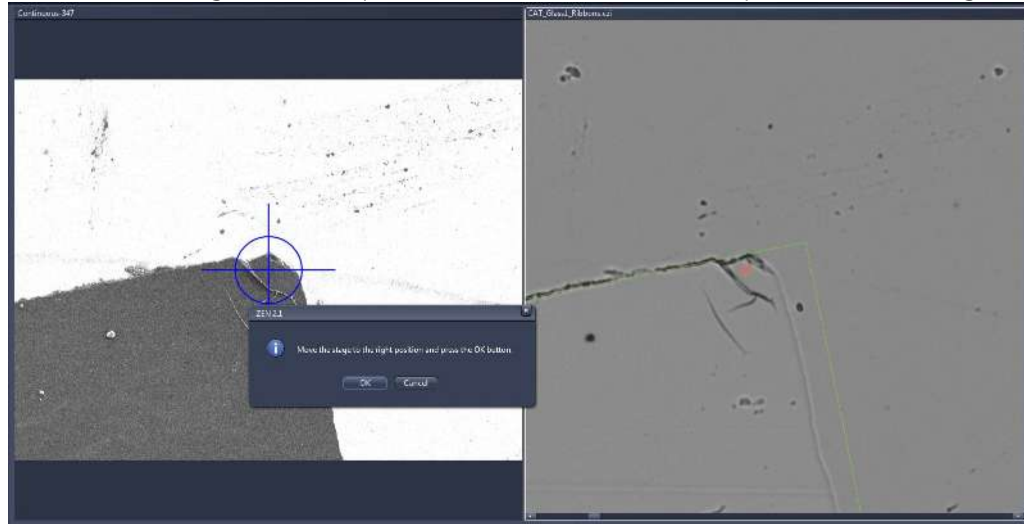
8. Move the correction points to prominent positions on the sample containing structures which are easy to recognize, e.g. the corner of the ribbons.



9. Click on **Set correction offset**. Note that for setting the offset correction you should use the same magnification which will be used for image acquisition later.
   → The stage moves to the first correction point.
   → In the left image you will see the SEM image position. In the right image you will see the correction point on the LM image. The positions do not match exactly.



10. Move the SEM stage so that its position will match the correction point in the LM image.



11. If the positions do match confirm the message by clicking **OK**.
    → The stage will move to the next correction point automatically.
    → Repeat the last 3 steps for the remaining correction points.
    → If you have finished the offset correction you should continue with verifying the support points again. As this is exactly the same procedure like described for the LM acquisition, read the chapters *Imaging the ribbons [282]* and *Imaging the ROIs [289]*. Note that you must use the control panel for the SEM to adjust the SEM parameter.
12. After you have verified the support points click on **Acquire**.

The ROIs will be imaged with the SEM now. Depending on your experiment settings this can take some time.

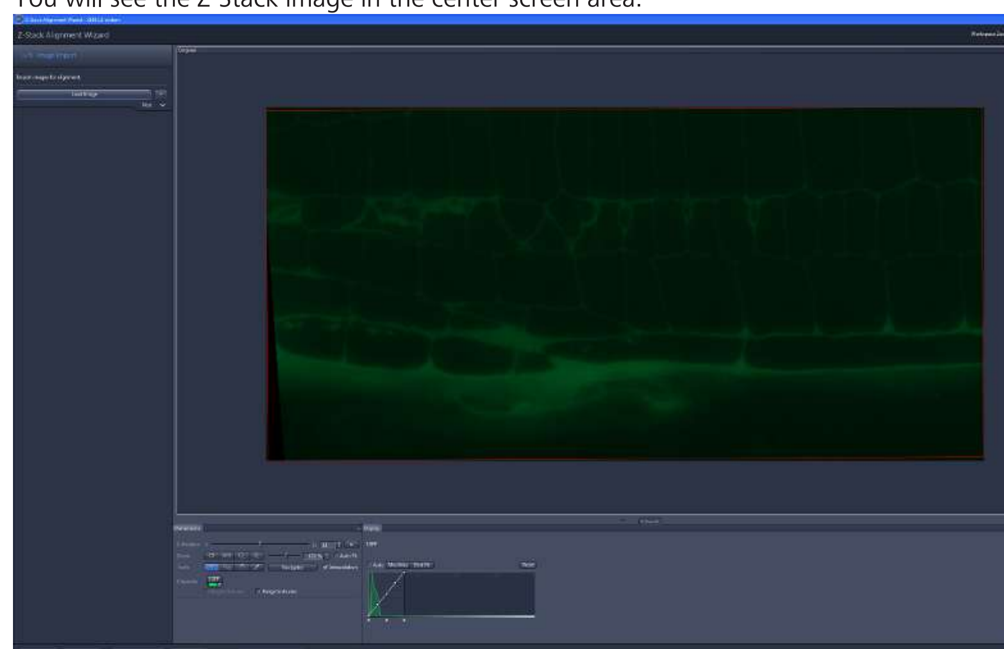
12.6.5.8 Aligning the Z-Stack Image

The wizard contains the following 6 steps:

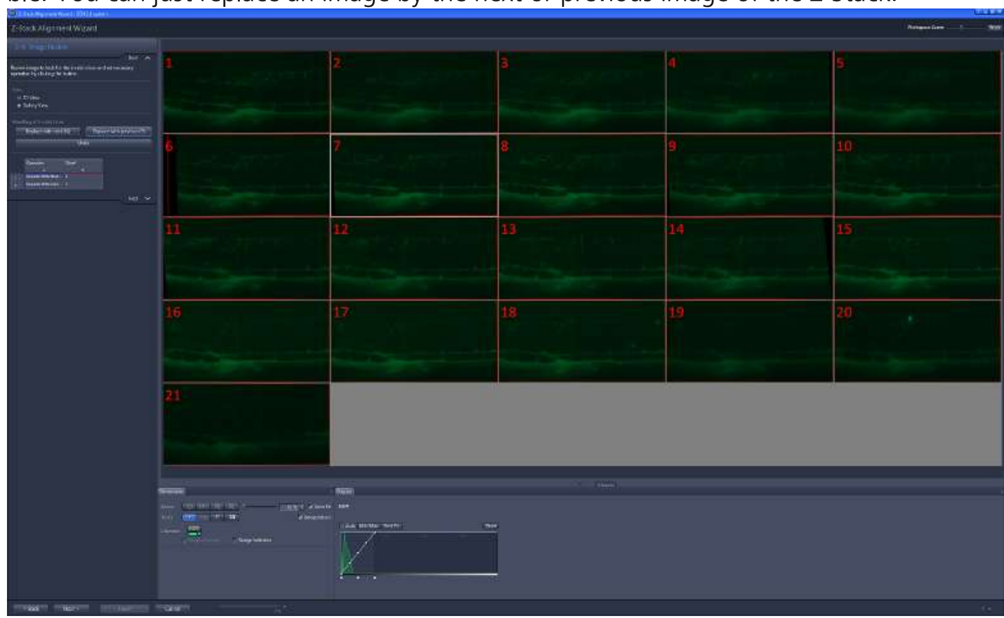
- Image Import
- Pre-Processing
- Image Review
- Alignment
- Manual Correction
- Final Image Creation

1. In the **CAT** tool click on **Start Z-Stack Alignment Wizard**.
   → You will see the first step **Image Import** of the wizard.
2. Click on **Load Image** and select the acquired Z-Stack image from the file system. In our example we choose the LM image. The same process has to be performed for the SEM image afterwards.

→ You will see the Z-Stack image in the center screen area.

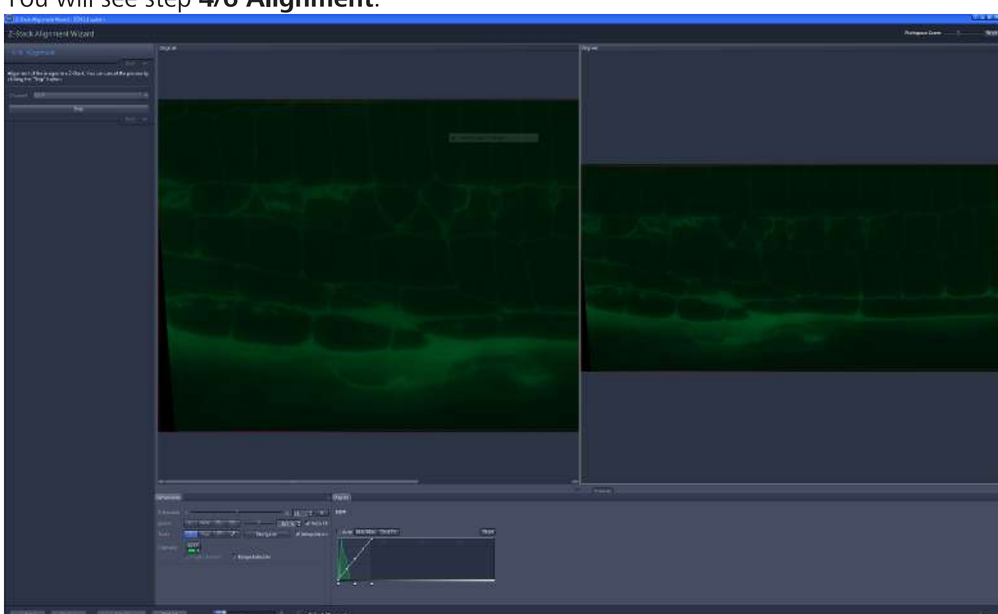


3. Click on **Next**.
   → You will see step **2/6 Pre-Processing**.
4. If your image is a tile image click on **Apply Stitching** to correct the offset between the individual tiles.
5. If your image is a SEM image click on **Histogram Equalization** to adjust the image display.
6. Click on **Next**.
   → You will see step **3/6 Image Review**. Note that in this step no image acquisition is possible. You can just replace an image by the next or previous image of the Z-Stack.



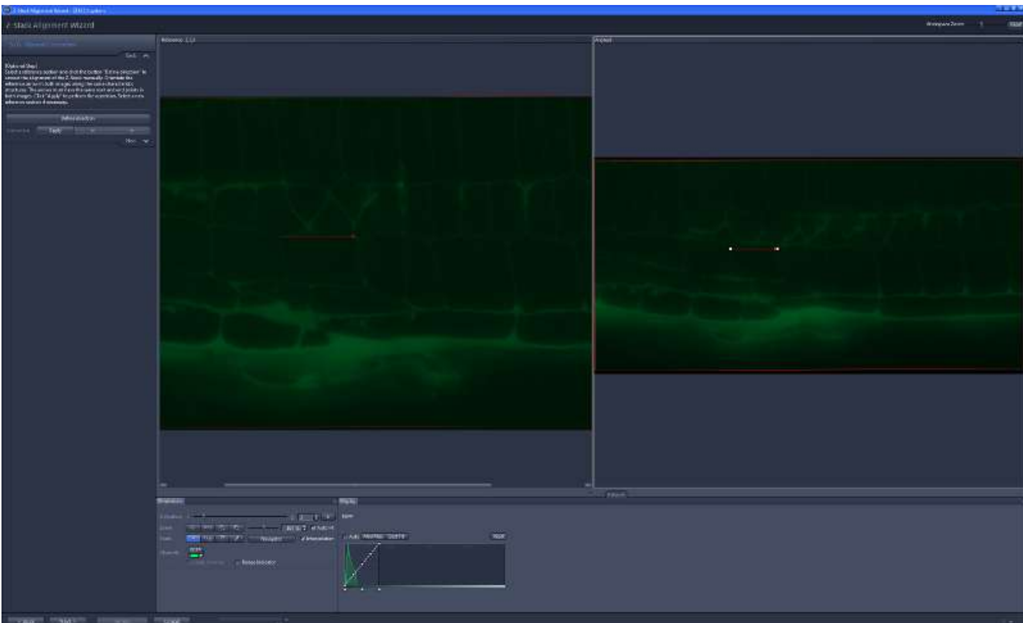
7. Select the image to be replaced by clicking on it with the mouse and click on **Replace with next** or **Replace with previous**. Alternatively you can press the *N* or the *P* key. Note that the table of the replaced image will not be saved.
8. Click on **Next**.

→ You will see step **4/6 Alignment**.



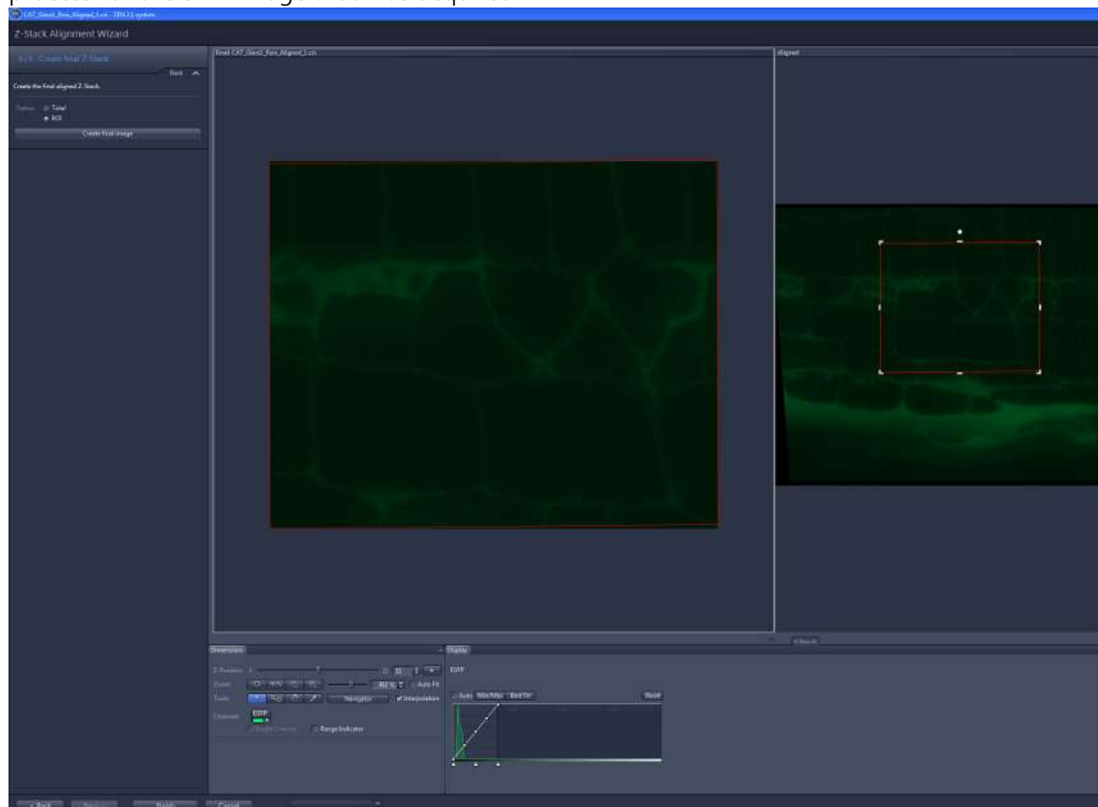
9. If you have acquired a multi-channel image select the reference channel from the **Channel** list.
10. Click on **Start Alignment**.
    → The alignment of the Z-Stack image will be performed automatically. After the alignment you will see the original Z-Stack image in the left image and in the right image you will see the aligned Z-Stack image.
11. Click on **Next**.
    → You will see step **5/6 Manual Correction**.
    → If you browse through the Z-Stack by using the Z-Position slider and you still realize a shift between the single Z-Stack images, you can perform a manual correction of the single Z-Stack images. Therefore continue as follows:
12. Click on **Define direction**.
    → A red arrow will appear in the right and in the left image.
13. Place the arrow in the left image at a prominent structure in the image which is easy to recognize through the full Z-Stack.
14. Select the right image and browse through the Z-Stack by using the Z-Slider on the **Dimensions** tab.

15. When you realize a shift in an image adjust the arrow in the right image so that it matches with the prominent structure marked with the arrow in the left image. Note that you have to check and adjust the arrow for each image of the Z-Stack which does not match the position.



16. Click on **Apply**.
    → The correction will be applied. If you want to correct the Z-Stack at another position, simply repeat the procedure with another prominent structure. Of course you can undo and redo actions by using the **Undo** / **Redo** buttons.
17. Click on **Next**.
    → You are in step **6/6 Create final Z-Stack**.
18. If you want to create the Z-Stack image from the complete image select **Total** and click on **Create final image**.
19. If you want to create the Z-Stack image from the marked ROIs, select **ROI**. Note that you can adjust the marked ROI in its size and position here. If you have marked more than one ROI you can switch between the ROIs by using the **Scene** slider on **Dimension** tab.
20. Click on **Create final image**.

You have successfully aligned and created the Z-Stack image. Of course you have to repeat the process for the SEM image that was acquired.



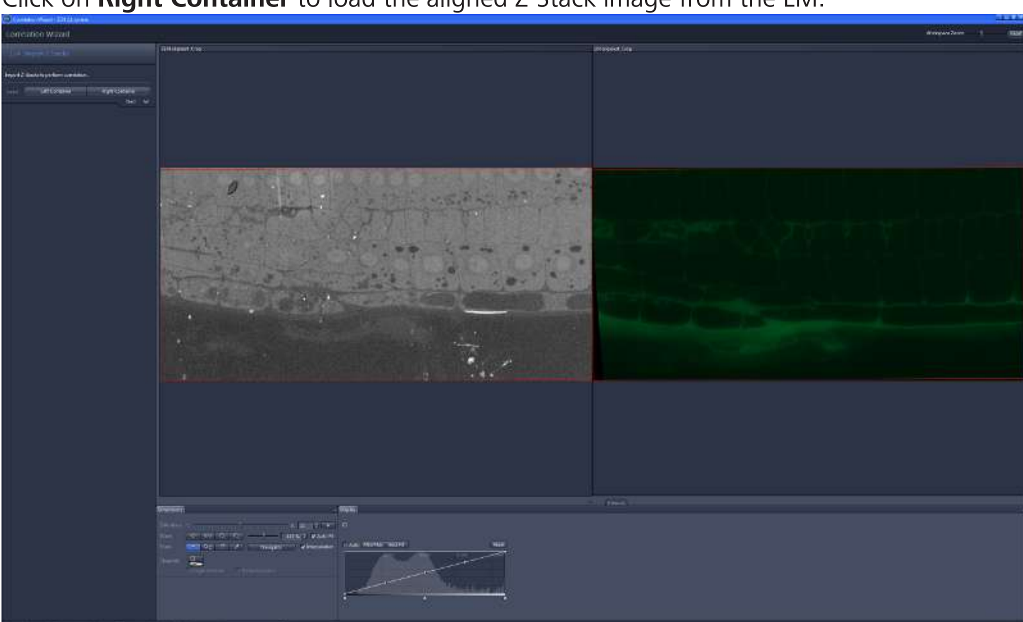
12.6.5.9 Correlating the LM and SEM images

The wizard contains the following 4 steps:

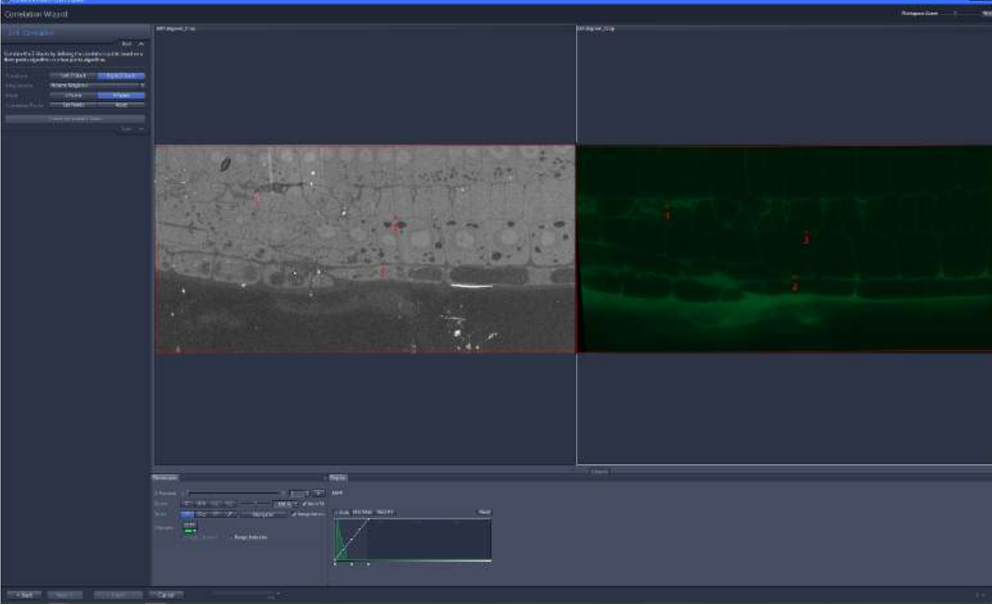
- Import Z-Stacks
- Correlation
- Manual Correction
- Create Final Correlation Image

1. In the **CAT** tool click on **Start Correlation Wizard**.
   → You will see the first step **Import Z-Stacks**.
2. Click on **Left Container** to load the aligned Z-Stack image from the SEM.

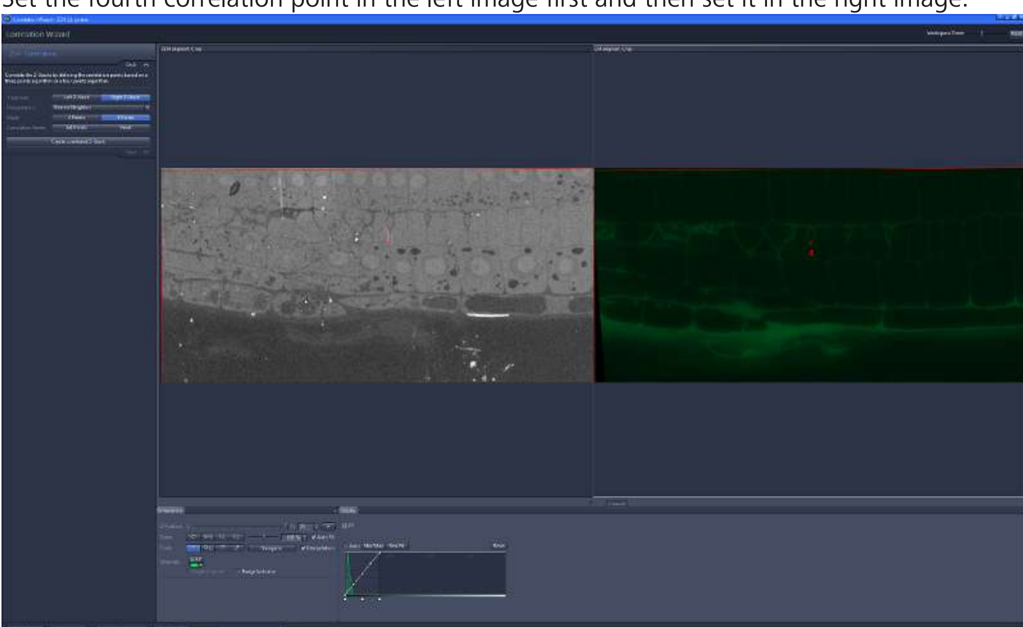
3. Click on **Right Container** to load the aligned Z-Stack image from the LM.



4. Click on **Next**.
   → You will see step **2/4 Correlation**.
5. Under **Transform** decide whether you want to transform the **Left Z-Stack** into the **Right Z-Stack** or vice versa.
6. Under **Mode** select **4 Points**.
7. Click on **Set Points**.
8. Set the first 3 correlation points in the first Z-Stack image of the left image.
9. Set the corresponding 3 correlation points in the first Z-Stack image of the right image.
   → After the third point in the right image was set, the software automatically jumps to the last Z-Stack image.

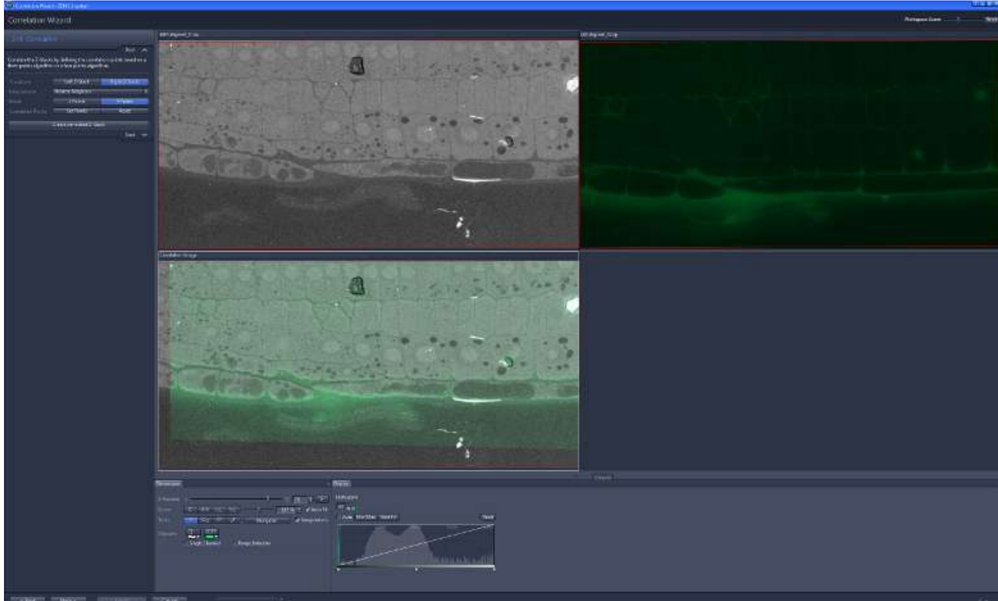


10. Set the fourth correlation point in the left image first and then set it in the right image.



11. Click on **Create correlated Z-Stack**.

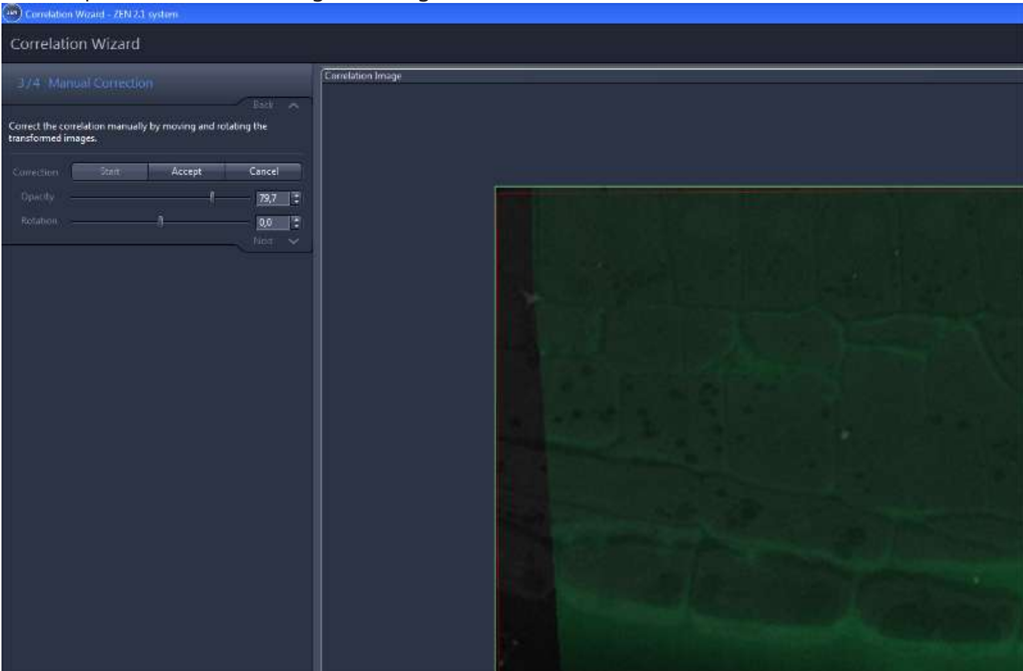
→ The correlated Z-Stack image will be generated and displayed in a separate image container.



12. Click on **Next**.

→ You will see step **3/4 Manual Correction**.

13. In this step you can manually correct the alignment of the images by moving and/or rotating the images according to each other. To rotate the image use the handle at the top of the image frame. To move the image simply left click on the image and hold the mouse button pressed while moving the image.

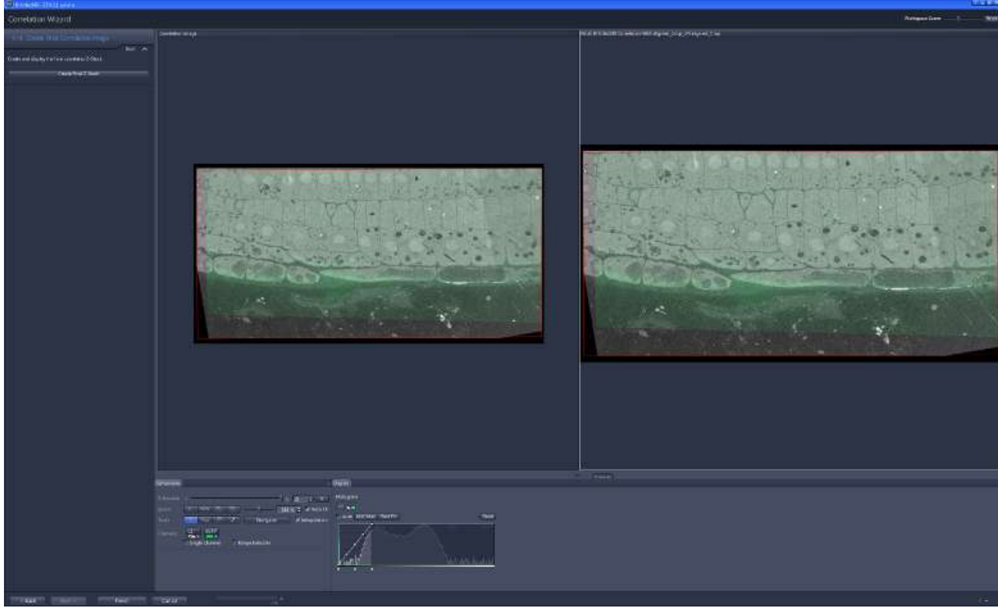


14. If you finished the alignment of an image click on **Accept**. You can browse through the correlated Z-Stack images by using the **Z-Position** slider in the **Dimension** tab.
15. Click on **Next**.

   → You will see step **4/4 Create Final Correlation Image**.

16. Click on **Create Final Z-Stack**.

   → The correlated Z-Stack image will be created.



17. Click on **Finish** to exit the wizard.

You have successfully created an correlated Z-Stack image.

12.6.6 Functions & Reference

12.6.6.1 CAT Tool

Using this tool you can calibrate and manage the sample holders and start the wizards which are used for acquiring images from serial sections, generating Z-stack images out of the single images and correlate two Z-Stack images from the light microscope (LM) and the scanning electron microscope (SEM).

Parameter	Description
Sample Name	Displays the name of the sample.
Select / Specify...	Opens the *Select Sample Dialog [▸ 303]*. There you can select a sample data sheet from the list or specify a new sample with user specific information. The specified sample information will be used for image processing (i.e. Z-Stack alignment) or for data management.
Sample Holder	Displays the name of the selected sample holder.
Select...	Opens the **Select Template** dialog. There you select the preferred sample holder or define new holder templates, see Selecting the sample holder.
Calibrate...	Opens the *Sample Holder Calibration Wizard [▸ 304]*. There you can calibrate the selected sample holder.
Apply to Image	Only visible if the **Show All** mode is activated. **Use this button only, when you forgot to calibrate the holder before you acquire the image.** Applies a calibration to an acquired image. Do not remove the sample from the correlative holder between image acquisition and calibration. Exception: correlative markers are on the sample holder.
Start Acquisition Wizard	Starts the **Acquisition Wizard**. For more information see *Acquisition Wizard [▸ 307]*.
Start Z-Stack Alignment Wizard	Starts the **Z-Stack Alignment Wizard**. For more information, see *Z-Stack Alignment Wizard [▸ 314]*.
Start Correlation Wizard	Starts the **Correlation Wizard**. For more information, see *Correlation Wizard [▸ 316]*.

12.6.6.1.1 Select Sample Dialog

Here you can select or create a sample data sheet.

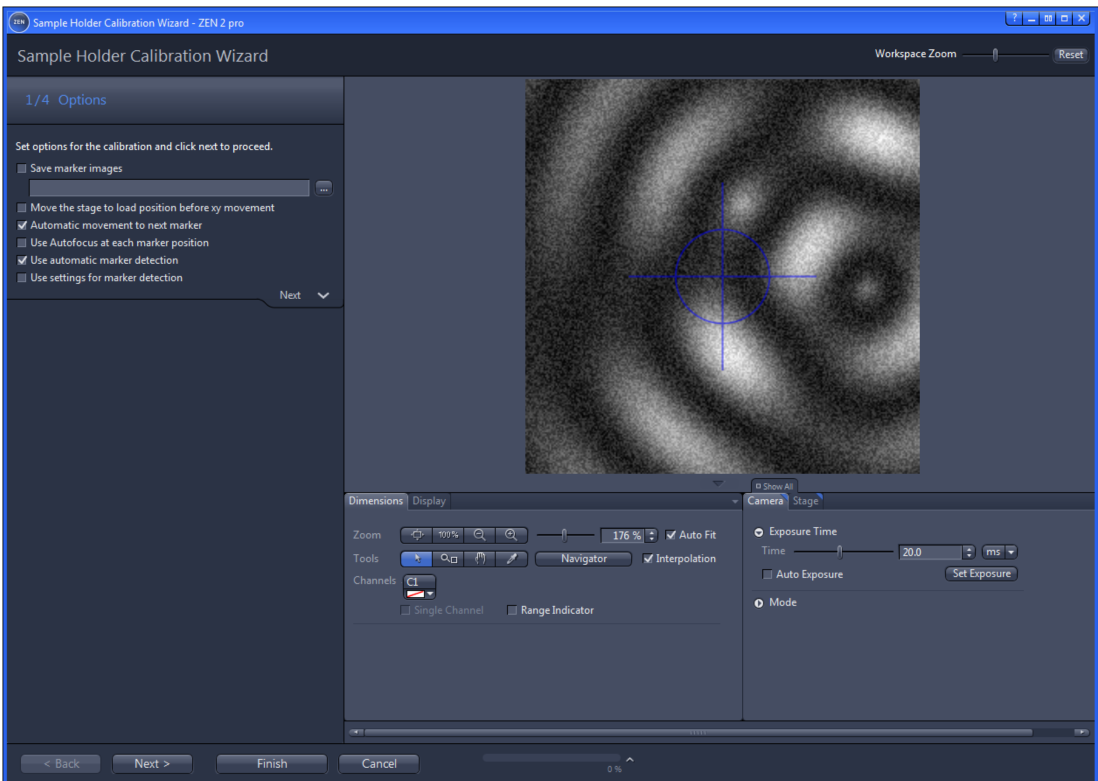
Parameter	Description
Folder	Shows the location, where the files are saved. If you click on the ... button you can change the storage location. The default path and folder is **C:\Users\user\Pictures\CAT Samples**.

Parameter	Description
	Within this folder, each sample is saved in a sub-folder. Images taken during image acquisition within the CAT Acquisition Wizard will be saved within the sub-folder, automatically. For a better clarity sub-folders with the name „[Date][Time]“ will be generated when another CAT run is started within the CAT acquisition wizard. In case the file name will exceed a certain number of characters the name will be shortened using the sign „°“.
List of specified samples	Shows the samples which are already specified within the software. If you select a sample in the list and click **OK**, the sample will be used in your experiment. If you click on the + button, the **New Sample** Dialog opens. There you can create a new sample definition which will be added to the list, see *Creating a new sample [▸ 275]*. If you click on the **Options** button, you will see further options for managing samples like **Show/Edit** or **Refresh Sample List**.
Information	Shows the specified sample information, e.g. name, description, number of sample carriers, sample carrier, and section thickness.

See also

CAT Tool [▸ 303]

12.6.6.2 Sample Holder Calibration Wizard



With the Sample Holder Calibration Wizard you calibrate the selected correlative sample holder. Make sure that you have selected the desired sample holder, see Selecting the sample holder.

12.6.6.2.1 Step 1: Options

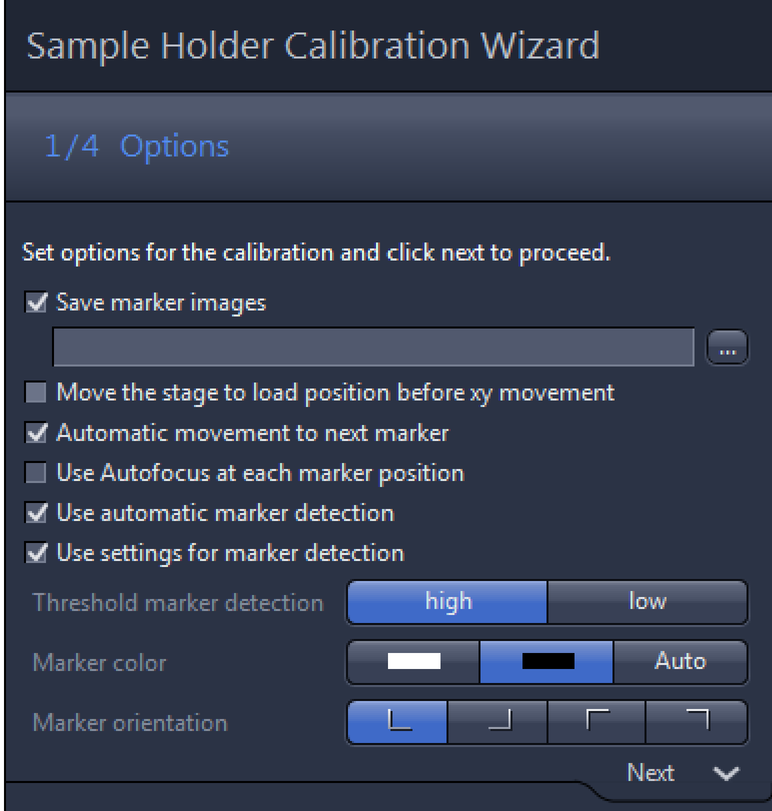


Fig. 14: Sample Holder Calibration Wizard Options

Option	Description
Save marker images	**Activated:** the marker images are saved during the calibration. The images can be used to check the calibration afterwards. Click on the **Select Folder (...)** button to select a storage folder.
Move the stage to load position before x/y movement	**Activated:** the stage will move to load position before moving to the next correlative calibration marker. In case of using an AxioObserver, the objective revolver moves to load position.
Automatic movement to next marker	**Activated:** By clicking on the **Next** button within the wizard the stage moves automatically to the next calibration marker. **Deactivated:** You must use the joystick to navigate to the markers. This is necessary when using a correlative holder which has no holder data deposited.
Use Autofocus at each marker position	This option is active only if the **Automatic movement to next marker position** checkbox is activated. **Activated:** the focus is adjusted automatically after moving to the next marker position.
Use automatic marker detection	**Activated:** The software will detect the small calibration marker automatically.

Option	Description
Use settings for marker detection	This option is active only if the **Use automatic marker detection** checkbox is activated. **Activated:** shows settings for marker detection (see description below). Here you select the properties of the calibration markers.

Settings for marker detection

Only visible if the **Use settings for marker detection** checkbox is activated.

Option	Description
Threshold marker detection: high – low	A low threshold for marker detection is used when the dimensions of the correlative L markers cannot be recognized precisely, e.g. when the sample holder is slightly filthy.
Marker color	Here you select the color of the markers displayed in the live image. **White:** the marker is displayed white on a dark background. **Black:** the marker is displayed dark on light background. **Auto:** the marker color is set automatically.
Marker orientation	Here you need to set the orientation of the L-markers on your sample holder. Click on the corresponding button to select the orientation of the calibration marker which you can see in the live image

If you click on the **Next** button you will move to the next step of the wizard.

12.6.6.2.2 Step 2-4: Calibration

In steps 2-4 of the wizard you will be guided through the calibration procedure.

Fig. 15: Sample Holder Calibration Wizard

Option	Function
Holder position	**Move to Position 1** button Moves the stage to marker position 1. This is possible only if the first position was set before and x/y coordinates are given. **Current** button Only visible for marker position 2 and 3. Moves the stage to the current marker position. This is possible only if the current position was set before and x/y coordinates are given.
Stage movement to the next marker	Here you can change the movement of the stage in x or y direction. This is necessary if during calibration the stage moves in the wrong direction.
Marker position	By clicking on the **Set** button, the actual marker position will be confirmed.

Click **Finish** to leave the wizard.

12.6.6.3 Acquisition Wizard

This wizard is used to image the serial sections or user-defined region of interest within the sections.

The steps **Overview Imaging**, **Ribbon Imaging**, **ROI Imaging** and **Re-Shoot** are image acquisition steps. The step **Re-Shoot** gives you the opportunity to image parts of the ROI-series or tiles of a tile image, later.

The wizard consists of 7 steps which are described in the following chapters:

- *1/7 Overview Imaging [307]*
- *2/7 Ribbon Specification (optional) [310]*
- *3/7 Ribbon Imaging (optional) [310]*
- *4/7 Section Specification [310]*
- *5/7 ROI Definition [313]*
- *6/7 ROI Imaging [313]*
- *7/7 ReShoot (optional) [313]*

12.6.6.3.1 Overview Imaging

In this step you can acquire an overview image that allows navigation on the sample. You will see the positions of the serial sections on the sample carrier. In general, for the overview image an objective with low magnification is used. This makes the acquisition fast due to a large field of view and limited number of tiles.

Image acquisition with objectives with higher magnification is possible. But keep in mind, that the number of tiles will increase due a smaller field of view as well as the acquisition time.

Info

We recommend to use phase contrast images for the acquisition. The used algorithm for the automatic section specification, see step 4, is most reliable then.

Parameter	Description
Image Acquisition / **Load Image**	By selecting the corresponding button you can decide whether to acquire an overview image or load an image.
Image Selection	If you have selected **Load Image**, a saved image file from the file system can be chosen. Therefore simply click on the ... button and navigate to your image file. The wizard will jump to the wizard step according to the information saved within the loaded image.
Experiment	If you have selected **Image Acquisition** you have to select an experiment from the **Experiment** list. Note that the experiment has to be set up and saved in advance, before you enter the wizard.
Objective	Here you can select the objective that you want to use for the acquisition of the overview image. As mentioned before, we recommend to use an objective with a low magnification (e.g. 2.5x or 5x).
Channels	Here you can select the channels that you want to use for the acquisition of the overview image. You can use more than one channel in one run, when your microscope is equipped with a motorized condenser.
Selected Light Source	Here you can select the light source that you want to use for the acquisition of the overview image. The light intensity can be adapted if a corresponding light source is selected.
Camera Settings	Here you can adapt the camera settings like changing the exposure time or activating / deactivating the shading correction. If a shading correction has been performed and activated in the selected experiment, the checkbox will also be activated automatically in the wizard.
Software Autofocus	Here you can activate the Software Autofocus functionality and apply it to the overview image. If activated, you can select the positions for focusing. During focusing no live image will be visible. Note that sensitive fluorescence labels might be bleached during the autofocus process.

Parameter	Description
Overview Image Definition	Here you can define the size of the overview image by defining a start and end position. Start End The software will calculate the area by means of the defined start and end position (= overview image). The number of tiles and the memory used will be displayed below the buttons.
- Set start position	Sets the current stage position as start position of the image area.
- Set end position	Sets the current stage position as end position of the image area.
- Move to start position	Moves the stage automatically to the defined start position. Note that the start position has to be defined before.
- Move to end position	Moves the stage automatically to the defined end position. Note that the end position has to be defined before.
No. of sample carrier	Only active, if you use more than one sample carrier for one correlative Z-Stack. Here you have to select the number of the used sample carrier. The total number of used sample carriers was defined in the CAT tool under **Select / Specify sample**.
Move the stage to load position before xy movement	**Activated:** the stage will move to load position before moving to the next correlative calibration marker. In case of using an AxioObserver, the objective revolver moves to load position.
Acquire Overview Image	Starts image acquisition. The acquisition can be stopped in between. Then the button will change to **Restart**. Before you restart the image acquisition, you can modify the settings. The status of the image acquisition is shown in the status bar of the software. After the overview image is taken, the image can be stitched. If you click on the **Apply Stitching** button stitching will be carried out.
Next	Brings you to the next wizard step.

See also

Ribbon Definition [▸ 310]

12.6.6.3.2 Ribbon Definition

This step is an optional step. It allows to image the ribbons with an objective with higher magnification, if necessary. E.g. when you would like to define the regions of interest by means of the sample structure that can only be identified using lenses with higher magnification.

Therefore you can mark the outlines of the ribbon on your sample. If you can`t see the sample structures, due to the overview image was acquired with a too low magnification, you can image your sample again using a higher magnification.

For marking the outlines in the image use the tools from the **Ribbon Definition** tab (e.g. Rectangle, Circle or Polygon).

See also

Ribbon Imaging [310]

12.6.6.3.3 Ribbon Imaging

For this step a split view will appear. On the left side you see the Live image. On the right side you see the overview image with the defined ribbons.

Info

To modify either the Live image or the image with the defined ribbons click on the corresponding container. The activated container will be marked with a white frame.

Again, like in step one for the overview image, you have to select the objective, channel, light source and adapt the exposure time. Additionally you have to generate a focus surface to ensure that your sample will be in focus during the image acquisition.

If you click on the **Create Ribbon Image** button, the ribbon image will be acquired.

See also

Section Specification [310]

12.6.6.3.4 Section Specification

To determine the positions of regions of interest (ROIs) within the sections, you have to define the sections. The section lines generate the reference system for the ROI positions. The sections are marked and outlined with a frame.

Info

Note that you must mark one section on each ribbon. Meaning when your sample has three ribbons, three sections have to be marked overall.

We recommend to use phase contrast images for the section specification. The algorithm used for the automatic section specification is most reliable when using phase contrast images. When bright field images are used, the algorithm might be sub-optimal. In that case you have the opportunity to add and to move the section contours manually.

On the **Section Definition** tab you will find tools and options for creating sections in the image.

Parameter	Description
Reference Section	
- Select	Activates the selection mode (default).
- Contour	If selecting this mode you can mark a contour line of a section.
- Keep	Keeps the selected tool active. You can then use the tool several times without interruption.
- Step Backward	Undo the last step
- Step Forward	Repeats the last step.
- Delete	Deletes a selected graphical element.
Section Index	Here you can determine the starting number of the ribbons. This is important when the ribbons of a sample are deposited on more than one sample carrier.
Selected Channel	Shows the selected channel which is used for the detection.
Detection Sensitivity	Here you can adjust the detection sensitivity from **Low** to **High** by using the slider. This will be done by modifying the contrast thresholds for the section detection algorithm. When setting a low sensitivity, sections will be recognized even if the contrast between the section and the substrate is low; disadvantage: sections will be recognized, even in areas where no serial sections are deposited. When setting a high sensitivity, the algorithm only recognizes sections, if there is a high contrast between section and substrate. If not all sections were recognized you have the possibility to copy section contours or to stamp section contours.
Section Detection	
- Apply	Starts the section detection on the sample. The software tries to detect each section within the ribbon.
Contrast Method	
- Auto	Auto is used by default. The system recognizes the contrast method of the image automatically.
- Ph.Contrast	Applies the phase contrast method. Even if you are using a brightfield image, phase contrast will be applied as contrast method.
- Brigthfield	Applies the brightfield contrast method. Even if you are using a phase contrast image, brightfield will be applied as contrast method.
Use Internal Structure	Activate this checkbox only when sample structures are clearly visible within the sections. If activated, sample structures are used for section detection, additional to contrast differences between sections and substrate. In case that sections are not detected properly, you have the possibility either to stamp section contours or to copy section contours.
Post Definitions	

Parameter	Description
- Stamp tool	If selecting this tool you can stamp in undetected sections after the section detection is finished. Therefore simply select the tool and move the mouse cursor in the area nearby the last detected section. The cursor will change to a stamp icon and you will be able to stamp in the missing section contours.
- Accept Ref. Section	If you click on this button reference contours will transform into section contours.

When the section detection is finished you have different options for sorting the sections according to your needs, if necessary. Therefore right click on the detected sections. You will see a context menu with the following sorting options:

Parameter	Description
Sort	▪ **Sort all sections in reverse order** Sorts all sections which have a section contour. The initial section with number 1 becomes the last section, the last section becomes the first section with the number 1 ▪ **Sort selected Ribbon elements in reverse order** Sorts the selected sections on a ribbon. The initial section with number 1 becomes the last section, the last section becomes the first section with the number 1
Copy selected sections from here	Copies the selected sections.
Copy selected Ribbon sections from here	Copies all section contours on the selected ribbon.
Paste Section(s) to here	Pastes the section contours (selection of certain section contours or all sections of a ribbon) to the selected position.
Exchange numbering of selected Ribbon sections	Exchanges the numbering of selected sections on different ribbons.
Highlight Related Ribbon Sections	Highlights selected sections on a ribbon.
Merge two Ribbon Sections	Merges sections on two different ribbons to one.

See also

ROI Specification [▸ 313]

12.6.6.3.5 ROI Specification

In this step you can screen your sample for interesting sample regions (ROIs) and mark this area by a graphical element. You can define several regions of interest within in one section.

On the **ROI Definition** tab you can draw either a rectangle, a circle or a freehand polygon/contour. Click on the **Apply** button to automatically identify the region of interest in all other sections. It is also possible to **Undo** / **Redo** an action using the corresponding buttons. To remove a graphical element select it and click on the **Delete** (bin icon) button.

Info

With the arrow keys on your keyboard you can jump from one ROI to the next ROI along the series to check if the structure of interest is still within the defined region of interest.

See also

ROI Imaging [▸ 313]

12.6.6.3.6 ROI Imaging

With this step you can image the ROIs which are detected and marked in the previous step. The tile images will be generated from all defined region of interests automatically.

Info

The size of the snapped tile images of a ROI series can change due to the number of tiles which are necessary to image the defined region of interest. The number of tiles can vary due to the bending of the ribbon.

See also

Re-Shoot [▸ 313]

12.6.6.3.7 Re-Shoot

This step is helpful, if some tiles or regions of interest are blurry. These tiles/regions can be replaced by repeating the acquisition of the selected tiles or tile images. The procedure is as follows:

- Select all blurry tiles, first.
- Adjust the focus for each tile position.
- Take new images.

Parameter	Description
Select Tiles	If this mode is active, you can select the tiles which you want to re-shoot. Use the **Z-Position** slider under **Dimension** tab or the arrows within the Image area to scroll through the acquired images. If you found a tile image that you want to re-shoot, simply click on it. Then the color of the image frame turns from red to green. Note that all tiles or blurry regions have to be defined, before the image acquisition can be repeated.

Parameter	Description
Acquire	If this mode is active you can acquire the selected tiles again after the focus was adjusted manually. If you click on this button, the stage will move to the first tile and the following buttons will appear:
- Snap	Acquires a new image.
- Replace	Replaces the old tile by the new tile.
- Correct Bright-ness	In case the tile is brighter or darker, here you have the possibility to adapt the brightness of the tiles image

Click **Finish** to exit the wizard.

12.6.6.4 Z-Stack Alignment Wizard

This wizard is used to align the single images of a Z-Stack image. The wizard consists of 6 steps which are described in the following chapters:

- *1/6 Image Import [▸ 314]*
- *2/6 Pre-Processing [▸ 314]*
- *3/6 Image Review [▸ 315]*
- *4/6 Alignment [▸ 315]*
- *5/6 Manual Correction (optional step) [▸ 316]*
- *6/6 Final Image Creation [▸ 316]*

12.6.6.4.1 Image Import

In this step you can load your acquired Z-Stack images which you want to align. Therefore simply click on the **Load** button and select the image file from the file system.

See also

Pre-Processing [▸ 314]

12.6.6.4.2 Pre-Processing

In this step you can perform pre-processing functions on the loaded image, e.g. Stitching (only for tile images), Brightness and Contrast Correction (only for SEM images).

Parameter	Description
Apply Stitching button	Only visible if a tile image is loaded. If you click on this button, stitching is performed automatically on the image. The stitching can be canceled (Undo) or repeated (Redo) by us-ing the arrow buttons.
Clip Limit	Reduces noise in the image. The higher the Clip Limit , the lower the noise. The clip limit can be adjusted between 0 and 10 %.
Region Size	Defines the region for histogram equalization. The smaller the area, the higher the contrast, but the noise will increase, too. The Region Size can be adjusted from 16 to 1024 px.

Parameter	Description
Histogram Equalization	If you click on this button, the SEM images are adapted to the selected values. The Histogram Equalization can be canceled (Undo) or repeated (Redo) by using the arrow buttons.

See also

Image Review [315]

12.6.6.4.3 Image Review

This step is used for reviewing the single images of a Z-Stack. This is necessary because certain images might not be useful for 3D reconstruction due to problems during the image acquisition or sample preparation issues (wrinkles or ruptures within the section). These regions can be replaced either by the previous image or by the following image. To review the images, the images can be displayed as single 2D images in the **2D** view or as images series in the **Gallery** view.

Parameter	Description
2D View	If selected, you can review the single images of a Z-Stack image by using the 2D view. You can use the **Z-Position** slider to navigate through the single images. To replace an image select the image and click whether on the **Replace with next** or **Replace with previous** button. If you click on the **Undo** button the last action performed will be undone.
Gallery View	If selected, you can review the single images by using the **Gallery** view. The single images of a Z-Stack image are displayed as an image gallery. If you found an image that does not meet your expectations, simply select the image and replace it by the next or previous image.

See also

Alignment [315]

12.6.6.4.4 Alignment

In this step you perform the image alignment. Therefore simply click on the **Start Alignment** button. To cancel the alignment click on the **Stop** button.

Info

Before you start the alignment, select one channel as reference channel (e.g. DAPI, because it stains the nucleus and the nucleus is a proper structure for performing alignment).

During alignment a splitter view is visible. In the left container you can see the original images, in the right container you can see the aligned images.

See also

Manual Correction [▸ 316]

12.6.6.4.5 Manual Correction

In this step (optional) you can navigate through the aligned images and check the result of the alignment.

In case the results are unsatisfactory, you have the possibility to correct the alignment of the images manually. Misalignment can occur, when no characteristic structures are visible within the images.

See also

Final Image Creation [▸ 316]

12.6.6.4.6 Final Image Creation

Last but not least, in this step you create the final image.

Parameter	Description
Total	If selected, the complete image will be used for the image creation.
ROI	If selected, only the ROI area will be used for the image creation.
Create final image button	Creates the final aligned Z-Stack image.

Click **Finish** to exit the wizard.

12.6.6.5 Correlation Wizard

This wizard is used to correlate a Z-Stack image from the Light Microscope (LM) with the Z-Stack image from the Scanning Electron Microscope (SEM). The wizard consists of 4 steps which are described in the following chapters:

- *1/4 Import Z-Stacks [▸ 316]*
- *2/4 Correlation [▸ 317]*
- *3/4 Manual Correction [▸ 318]*
- *4/4 Create Final Correlation Image [▸ 318]*

12.6.6.5.1 Import Z-Stacks

In this step you can import the aligned Z-Stack images from the LM and the SEM, e.g. the Z-Stack image from the LM in the left container and the Z-Stack image from the SEM in the right container.

If you click on the **Left Container** button, the image is opened in the left image container.

If you click on the **Right Container** button, the image is opened in the right image container.

See also

Correlation [▸ 317]

12.6.6.5.2 Correlation

In this step you correlate the images.

Parameter	Description
Transform	Here you select which Z-Stack will be transformed. During transformation a pixel in the overlay image of the Z-Stack is calculated by using pixels of the two original images that shall be overlaid / merged.
Interpolation	Here you can select one of the following interpolation methods:
- Nearest Neighbor	The gray value of the resulting pixel in the overlay image is made of a pixel which is located next. This interpolation method is very fast.
- Linear	The resulting or calculated pixel in the overlay image is assigned to a gray value, which is the result of a linear combination of gray values derive from pixels located nearby (in the original image).
- Cubic	The calculated pixel in the overlay image is assigned to a gray value, which is calculated by means of a polynomial function using gray values of pixels in the original images; these pixels are located nearby the calculated pixel.
Mode	Here you can choose an algorithm mode:
- 3-Points	If selected, this mode enables you to set 6 correlation points after clicking on the **Set Points** button (3 points in each Z-Stack in each container)
- 4-Points	If selected, this mode enables you to set 8 correlation points after clicking on the **Set Points** button (4 points in each Z-Stack), 3 points in the first z-section, the last point in the last section
Correlation Points	If you click on the **Set Points** buttons you can set the correlation points. The number of correlation points is according to the selected algorithm. The cursor will change to a pipette symbol. Simply click in the image to set the points. Start with setting the first three points in the left container then set the corresponding correlation points in the right container. If a correlation point is set, a check mark icon will appear in front of the corresponding point. When you select the 4-Points-Algorithm the display will move automatically to the last image of the Z-Stack. Set the fourth correlation point in both containers. Make sure that the positions in both Z-Stacks are identical. After you have set all correlation points the cursor will be changed backwards from the pipette to the arrow. **Reset** deletes all correlation points in the image.
Create correlated Z-Stack	If you click on this button, the correlated Z-Stack will be generated and opened in a new image container.

See also

Manual Correction [▸ 318]

12.6.6.5.3 Manual Correction

In this step you can correct the correlation manually by moving and rotating the transformed image.

Therefore simply click on the **Start** button. Then you can interactively move the image by dragging and dropping it with the mouse or rotate the image by clicking on the circle button attached on top of the green image frame or using the **Rotation** slider. You can also change the image opacity by adjusting the corresponding slider.

If you click on the **Accept** button the manual correction will be adopted to the correlated image.

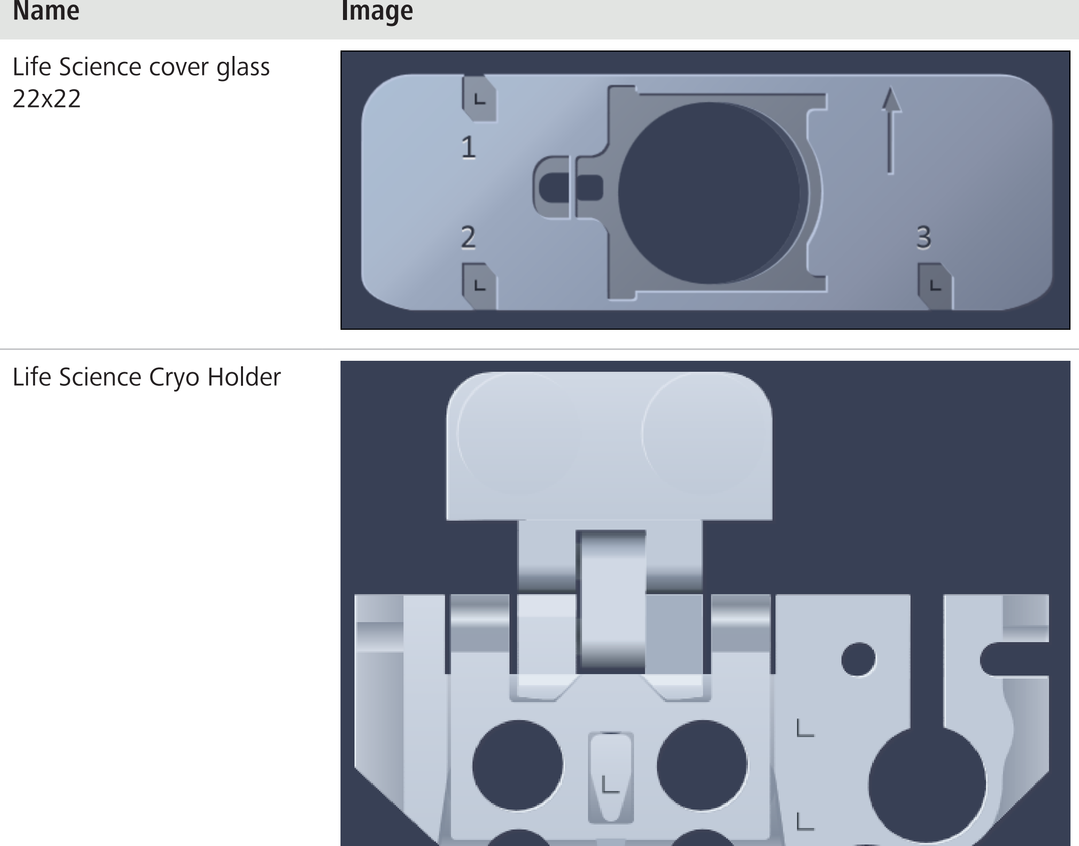
See also

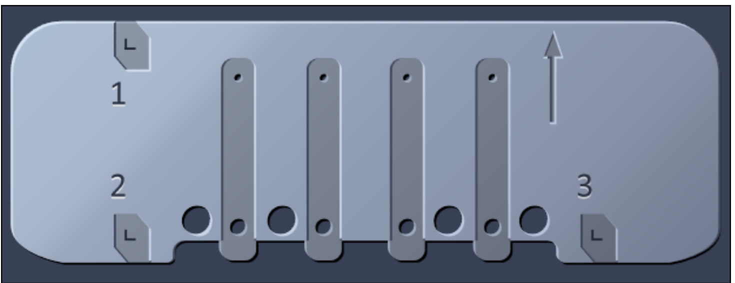
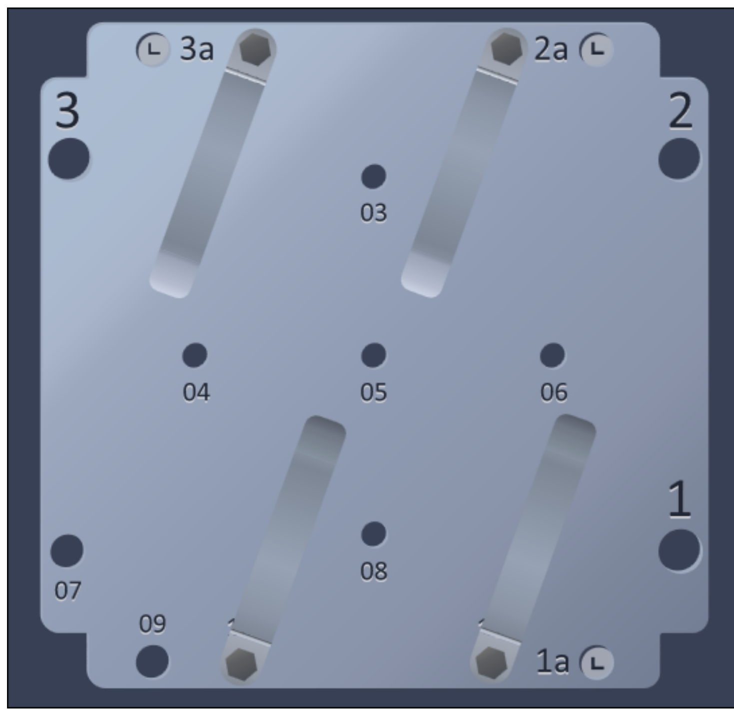
Create Final Correlation Image [▸ 318]

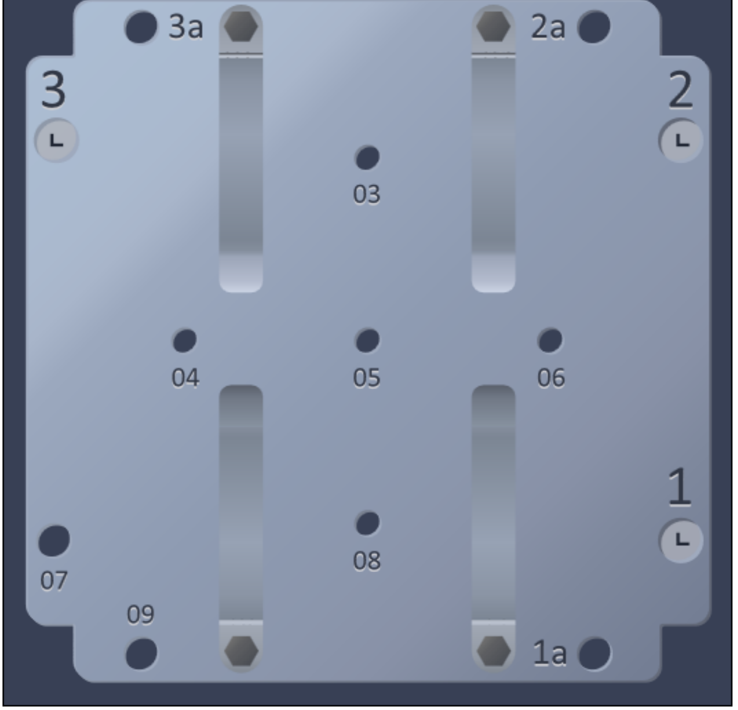
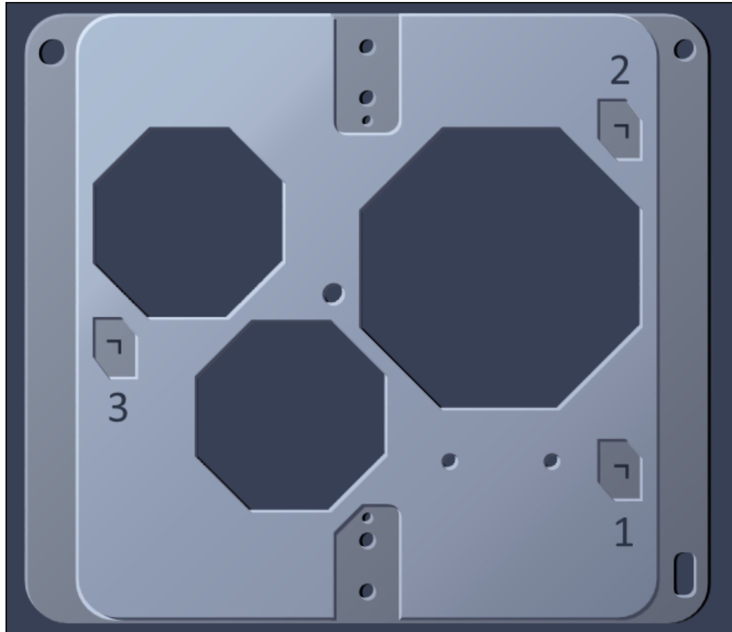
12.6.6.5.4 Create Final Correlation Image

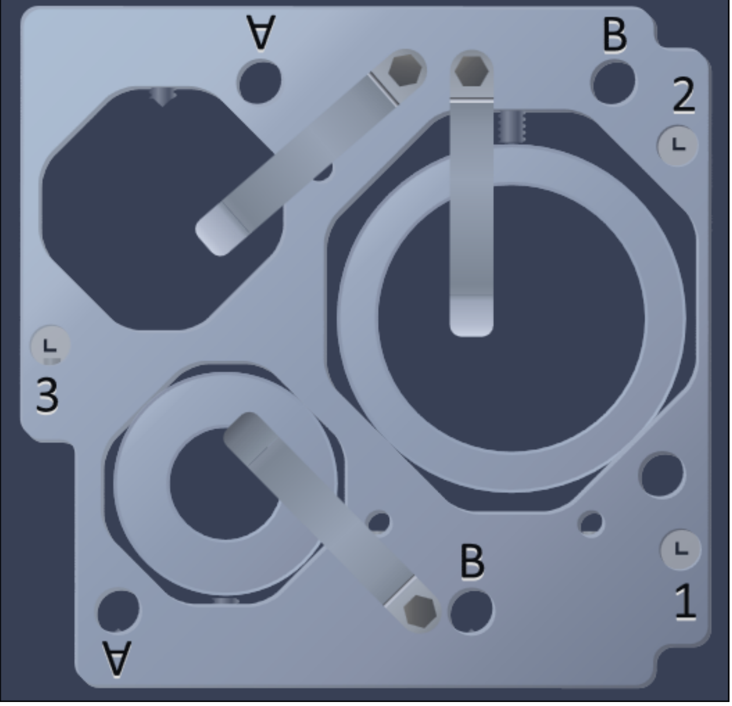
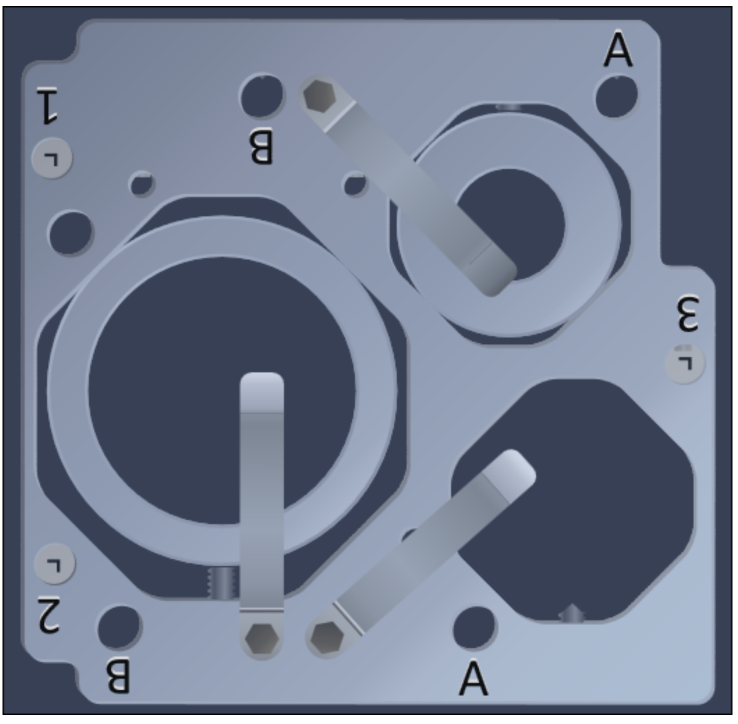
In this step create the final correlation image. Therefore simply click on the **Create final Z-Stack** button. Click **Finish** to exit the wizard.

12.6.6.6 Correlative Sample Holders

Name	Image
Life Science cover glass 22x22	
Life Science Cryo Holder	

Name	Image
Life Science for TEM Grids	
Cover glass with fiducials 22 x 22	
MAT Flat Stubs A	

Name	Image
MAT Flat Stubs	
MAT Universal A	

Name	Image
MAT Universal B_A	
MAT Universal B_B	

12.7 Deconvolution

This module offers you wide range of parameters, which allow to adapt the deconvolution to the sample conditions. The downside of this fact is, that using these parameters can be a challenge. The following functions are available for deconvolution in ZEN and are described in the following chapters:

- **Deconvolution (defaults):** Four methods are available and are automatically adapted to the type of instrument, which was used to acquire the image. This is the easiest to use deconvolution function
- **Deconvolution (adjustable):** Offers access to all available function parameters and therefore provides the necessary flexibility for demanding samples and sample conditions.
- **PSF Wizard:** this function offers a wizard which guides the user through a series of steps to create a PSF from a z-stack image of multiple fluorescent beads. This function is recommended to create experimentally measured PSF's.

12.7.1 Basics of Deconvolution

Microscopy creates images of objects which should represent the nature of the object as well as possible. Fluorescent light, which emanates from the object, passes through the various optical elements of the beampath and eventually gets collected by the detector. Unfortunately, on the way to the detector the signal is changed in such a way, that the quality of the resulting image suffers. As a consequence, the image is never a 100% correct representation of the object. This effect is strongest seen in classical widefield fluorescence microscopy which does in fact not offer any optical sectioning capability, but also exists to a different degree in optical sectioning microscope systems, e.g. Confocal, Lightsheet or ApoTome.

Fortunately, the dominant function which has this deleterious effect on the image, is based on the optical design principles of the light microscope and therefore well understood. We call this the point spread function (PSF) of the microscope system.

Deconvolution is a mathematical method which can reverse the effect of the PSF on the image and can therefore to a large extent restore the image to better represent the object. In the case of widefield imaging, Deconvolution can even convey optical sectioning properties to the result image allowing true three-dimensional restoration.

The following improvements can be obtained by using deconvolution:

- Denoising
- Removal of out of focus light -> deblurring, improved contrast
- Increasing signal to noise ratio by reassigning photons
- Restoration of sparsely sampled data
- Increase of resolution in X, Y and Z

As the object and the way, it was prepared, becomes part of the optical system during imaging, the largest variable to consider when doing deconvolution is the sample itself. Since the sample conditions can vary widely, information about the sample needs to be provided to the deconvolution function. The better the sample conditions are known, the better the outcome will be.

12.7.2 Deconvolution (defaults)

This method allows you to use 4 different algorithms for deconvolution, without any further settings.
The following algorithms are provided in the **Parameter** tool:

Parameter	Description
Simple, very fast (Nearest Neighbor)	Executes the fast Nearest Neighbor method using default parameters.
Better, fast (Regularized Inverse Filter)	Executes the Regularized Inverse Filter algorithm for image enhancement.
Good, medium speed (Fast Iterative)	Executes the Fast Iterative restoration method.
Excellent, slow (Constrained Iterative)	Executes the Constrained Iterative quantitative restoration method.

12.7.3 Deconvolution (adjustable)

Deconvolution can be done either by using the computers CPU or by using a graphics card. Using a graphics card can speed up deconvolution processing quite dramatically. A NVIDIA graphics card is required which supports the CUDA processing library. Contact your sales representative for further details about supported graphic card models.

For a detailed overview of all deconvolution methods (and its combinations) see *Deconvolution Methods in ZEN [▸ 337]*. This method allows you to use and individually configure 4 different algorithms for deconvolution (short DCV). Under **Parameters** two tabs are available for detailed configuration:

- On the **Deconvolution** tab, you can select the desired algorithm and define the precise settings for it, see *Deconvolution tab [▸ 323]*.
- On the **PSF Settings** tab, you can see and change all key parameters for either generating a theoretically calculated PSF, or selecting an experimentally measured PSF, see *PSF Settings tab [▸ 329]*.

12.7.3.1 Deconvolution tab

Info

Expert knowledge is required for some of the settings. If you are in doubt, leave the settings unchanged.

Algorithm dropdown list

Here you can select the algorithm that is used. The following algorithms are available:

- Nearest Neighbor
- Regularized Inverse Filter
- Fast Iterative
- Constrained Iterative

Enable Channel Selection

Activated: Applies the settings on a channel-specific basis. This allows you to set parameters for each channel individually. You will see a separate, colored tab for each of the channels.

Deactivated: Applies the same settings to all channels of a multichannel image.

12.7.3.1.1 Normalization section

Here you can specify how the data of the resulting image are handled:

Parameter	Description
Clip	Sets negative values to 0 (black). If the values exceed the maximum possible gray value of 65636 when the calculation is performed, they are limited to 65636 (pixel is 100% white). Results from different input images can be quantitatively compared with each other.
Automatic	Normalizes the output image automatically. In this case the lowest value is 0 and the highest value is the maximum possible gray value in the image (gray value of 65636). The maximum available gray value range is always utilized fully in the resulting image. Results from different input images cannot directly be compared quantitatively with each other.

12.7.3.1.2 Set Strength Manually section

If you have selected the **Nearest Neighbor** algorithm, the checkbox is always activated.

Info

If you have selected the **Fast Iterative** algorithm, the checkbox is also always activated. Using the slider you can then enter the number of iterations used directly, as, in contrast to the other methods, no regularization is performed.

Activated: Enter the desired degree of restoration using the slider.

To achieve strong restoration and best contrast, move the slider towards **Strong**.

To achieve lower restoration but smoother results, move the slider towards **Weak**.

If the setting is too strong, image noise may be intensified and other artifacts, such as "ringing", may appear.

Deactivated: Determines the restoration strength for optimum image quality automatically. This is recommended for widefield and confocal images and is therefore deactivated by default.

The restoration strength is inversely proportional to the strength of so-called regularization. This is determined automatically with the help of Generalized Cross Validation (GCV).

12.7.3.1.3 Convergence History

Only visible if the **Show All** mode is activated.

This section is only visible if you have selected the **Fast Iterative** or **Constrained Iterative** algorithm.

The progress of the calculation is displayed here as line graph. Several quality parameters are measured for each iteration and once either an optimum or the maximum allowed number of iterations is reached, the processing is stopped. This display allows you to observe directly how the iterative method affects the available data. It also shows how many iterations have been used and how much time is being used per iterations.

12.7.3.1.4 Corrections section

To show the section in full, click on the **arrow** button .

Parameter	Description
Lamp Flicker	**Activated:** Analyzes the total brightness of each Z-plane. In the event of non-constant deviations in the total brightness between neighboring planes, a compensation factor is taken into account. Activate this function if you have acquired your images using an old fluorescent lamp that exhibits strong fluctuations/flickering in brightness.
Fluorescent Decay	**Activated:** Corrects bleaching of the sample during acquisition of the Z-stack. This function should only be activated for widefield images. Use it if your sample undergoes strong bleaching during acquisition.
Background	**Activated:** Analyzes the background component in the image and removes it before the DCV calculation. This can prevent background noise being intensified during DCV.
Bad Pixel Correction	**Activated:** Employs a fully automatic detection and removal of spurious or hot pixels (also known as stuck pixels) in an image stack which might interfere with the deconvolution result. It is based on the analysis of the gray level variance in the neighborhood of each pixel in the image. It is recommended to use this parameter only, if stuck pixels are observed in the input image.

12.7.3.1.5 Advanced Settings section

Only visible if the **Show All** mode is activated.

This section is only visible if you have selected the **Inverse Filter**, **Iterative (Fast)** or **Iterative** algorithm.

To show the section in full, click on the **arrow** button .

Depending on which algorithm you have selected, different advanced setting options are available. The relevant settings are described in the following chapters for each algorithm:

12.7.3.1.6 Advanced settings (Regularized Inverse Filter)

Regularization

Here you can select which frequencies in the image are taken into account during regularization:

Parameter	Description
Regularization	
- Zero Order	Regularization based on G-difference, modeled on Tikhonov, but accelerated.
- First Order	Regularization based on Good's roughness. Under certain circumstances, more details are extracted from noisy data. May be better suited to the processing of confocal data sets.
GPU Acceleration	Only available, when having installed a suitable (NVIDIA, CUDA based) graphics card. The checkbox is then activated by default. If unchecked, CPU processing is being used instead.

12.7.3.1.7 Advanced settings (Fast Iterative)

Parameter	Description
Likelihood	Here you can decide which likelihood calculation you want to work with:
- Poisson (Meinel)	The calculation according to Meinel works with one convolution per iteration and converges very fast, normally in 4-5 iterations. This method can also produce artifacts, however.
- Poisson (Richardson-Lucy)	The calculation according to Richardson-Lucy, on the other hand, normally requires hundreds of iterations and therefore takes considerably longer. This method is, however, somewhat more robust producing less artifacts. Precondition is however, that the PSF is known very well.
Regularization	For the **Poisson (Meinel)** calculation it is also possible to perform **zero order** (G-difference) regularization here as an option. This means, however, that the calculation will take considerably longer and the main advantage of the greater speed of Meinel is lost.
- None	No regularization is performed.
- Zero Order	Regularization based on G-difference, modeled on Tikhonov, but accelerated.
- Total Variation	Total Variation regularization denoises the input data but protects the edges of structures. This can improve results for noisy data but increases the computation time quite significantly.
Optimization	
- Numerical Gradient	If selected, an attempt is made to determine the trend of the iterations in advance and extrapolate this to the entire calculation. This can significantly speed up the calculation.

Parameter	Description
First Estimate	
- Input Image	The input image is used as the first estimate of the target structure (default).
- Last Output Image	The result of the last calculation is used to estimate the next calculation. This can speed up a calculation that is repeated using slightly different parameters.
- Mean of Input Image	No estimate is made, the mean gray level of the input image is being used. This is the most rigid application of deconvolution. It should be chosen for confocal images, where the data sampling can be quite sparse. The computation time will increase, but missing information can be recovered from the PSF.
Maximum Iterations	Here you can indicate the maximum permitted number of iterations that you want. In the case of Richardson-Lucy, you should allow significantly more iterations here.
Quality Threshold	Defines the quality level at which you want the calculation to be stopped. The percentage describes the difference in enhancement between the last and next-to-last iteration compared with the greatest difference since the start of the calculation. 1% is the default value. Lowering this can bring about small improvements in quality.
GPU Acceleration	Only available, when having installed a suitable (NVIDIA, CUDA based) graphics card. The checkbox is then activated by default. If unchecked, CPU processing is being used instead.
GPU Tiling	Only available for very large images which exceed the available graphic card memory. With this function the image is split up in smaller portions which fit into the memory of the graphic card. The function automatically determines into how many tiles the image must be split to allow maximum usage of the graphics card. The resulting tiles will automatically be stitched together for the final output result. If deactivated, tiling will not be done, however, in this case only certain sub-functions of deconvolution can run on the graphics card and the speed increase compared to CPU processing will be lower. The image quality might be higher than with tiling because there is no need for stitching.

12.7.3.1.8 Advanced settings (Constrained Iterative)

Parameter	Description
Likelihood	Here you can decide which likelihood calculation you want to work with:
- Poisson	Calculation according to Poisson, this is normally the correct noise model for microscopic images.
- Gauss	Calculation according to Gauss. If detector noise is dominant over sample noise, using a Gaussian noise model can be advantageous, however, this is rarely the case with modern microscopy systems.

Parameter	Description
Regularization	Here you can enter which frequencies in the image are taken into account during regularization:
- Zero Order	Regularization based on G-difference, modeled on Tikhonov, but accelerated.
- First Order	Regularization based on Good's roughness. Under certain circumstances, more details are extracted from noisy data. This regularization can sometimes produce better results for the processing of confocal data sets.
- Second Order	Regularization according to Tikhonov-Miller. Here higher frequencies are penalized more than in the case of Good's roughness. Results have a tendency to become overly smoothed.
Optimization	
- Analytical (Newton Raphson)	Here an attempt is made to optimize the iterations analytically. This option is usually faster but may also be somewhat less precise.
- Line Search	Searches rigorously and comprehensively for the minimum. It is therefore more robust, but the calculation takes longer. Line search is recommended for confocal data sets especially, if they are noisy as this can enforce convergence even for noisy and sparsely sampled data.
First Estimate	
- Input Image	The input image is used for the first estimate of the target structure (default).
- Last Output Image	The result of the last calculation is used to estimate the next calculation. This can speed up a calculation that is repeated using slightly different parameters.
- Mean of Input Image	No estimate is made for the next iteration. This is the most rigid application of deconvolution. It should be chosen for confocal images, where the data sampling can be quite sparse. The computation time increases increase, but missing information can be recovered from the PSF.
Maximum Iterations	Here you can indicate the maximum permitted number of iterations that you want.
Quality Threshold	Defines the quality level at which you want the calculation to be stopped. The percentage describes the difference in enhancement between the last and next-to-last iteration compared with the greatest difference since the start of the calculation. Lowering this can bring about small improvements in quality.
GPU Acceleration	Only available, when having installed a suitable (NVIDIA, CUDA based) graphics card. The checkbox is then activated by default. If unchecked, CPU processing is being used instead.

Parameter	Description
GPU Tiling	Only available for very large images which exceed the available graphic card memory. With this function the image is split up in smaller portions which fit into the memory of the graphic card. The function automatically determines into how many tiles the image must be split to allow maximum usage of the graphics card. The resulting tiles will automatically be stitched together for the final output result. If deactivated, tiling will not be done, however, in this case only certain sub-functions of deconvolution can run on the graphics card and the speed increase compared to CPU processing will be lower. The image quality might be higher than with tiling because there is no need for stitching.

12.7.3.2 PSF Settings tab

All key parameters for generating a theoretically calculated Point Spread Function ("PSF") are displayed here.

Info

Ordinarily, images that have been acquired using **ZEN** (of the *.czi type) automatically contain all microscope parameters, meaning that you do not have to configure any settings on this page. Most parameters are therefore grayed out in the display. It is possible, however, that as a result of an incorrect microscope configuration values may not be present or may be incorrect. You can change these here. The correction of spherical aberration can also be set here.

12.7.3.2.1 Microscope parameters section

The most important microscope parameters for PSF generation that are not channel-specific are displayed in this section.

Info

If you enter incorrect values, this can lead to incorrect calculations. If the values here are obviously wrong or values are missing, check the configuration of your microscope system.

Parameter	Description
Microscope drop-down list	Displays which type of microscope has been used. There are two main options: conventional microscope (also known as a widefield microscope) and confocal microscope, for which the additional pinhole diameter parameter applies.
NA Objective	Displays the numerical aperture of the objective.
Immersion	Displays the refractive index of the immersion medium. Please note that this can never be smaller than the numerical aperture of the objective. You can make a selection from typical immersion media in the dropdown list next to the input field.
Scale lateral	Displays the geometric scaling in the X/Y direction.

Parameter	Description
Scale axial	Displays the geometric scaling in the Z direction.
Override button	To change the input fields that are normally grayed out, click on the button. The input fields and dropdown lists are now active. The text on the button then changes to **Reset**. To restore the original values saved in the image, click on the **Reset** button.
Master Reset	Resets the metadata to the values which were originally stored in the image at time of acquisition. It reverts any changes made by pressing the **Override** button.

12.7.3.2.2 Advanced section

Only visible if the **Show All** mode is activated.

To show the section in full, click on the **arrow** button .

Phase Ring dropdown list

If you have acquired a fluorescence image using a phase contrast objective, the phase ring present in the objective is entered here. This setting has significant effects on the theoretical Point Spread Function ("PSF").

PSF generation dropdown list

There are two models for calculating the PSF:

Parameter	Description
Scalar Theory	The wave vectors of the light are interpreted as electrical field = intensity and simply added. This method is fast and is sufficient in most cases (default setting).
Vectorial Theory	The wave vectors are added geometrically. However, the calculation takes considerably longer.

Z-Stack

This field can only be changed if it was not possible to define this parameter during acquisition, e.g. because the microscope type was unknown. It describes the direction in which the Z-stack was acquired. Note that this setting is only relevant, if your are using the spherical aberration correction.

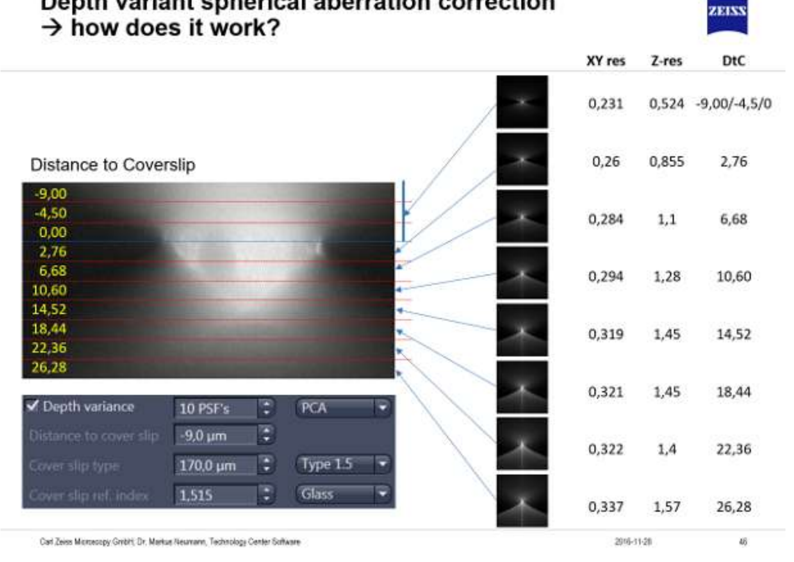
Parameter	Description
Descending	The Z-Stack descends away from the objective.
Ascending	The Z-Stack ascends towards the objective.

12.7.3.2.3 Aberration correction section

Only visible if the **Show All** mode is activated.

Here you can select whether you want spherical aberration to be taken into account and corrected during the calculation of the PSF. As with the other PSF parameters, most values are extracted automatically from the information about the microscope that is saved with the image during acquisition. The input option is therefore inactive.

Parameter	Description
Enable Correction	**Activated:** Uses the correction function. All options are active and can be edited.
Embedding medium	Here you can select the embedding medium used from the list.
Refractive index	Displays the refractive index of the selected embedding medium. Enter the appropriate refractive index if you are using a different embedding medium.
Manufacturer	Displays the manufacturer, if known.
Depth variance	When Aberration correction is activated, it is also possible to enable the creation of depth variant PSF's. This method allows for dramatic improvements in image restoration of thicker samples by creating axially variant theoretical PSF's as a function of the distance to the coverslip and the refractive index of the mounting medium. To use depth variant aberration correction activate the checkbox. In the spin box edit field you can define how many PSF's should be generated. The more PSF's you create, the better the results, but choosing many PSF's will increase the processing time. You should choose at least 3 PSF's. From the dropdown list you can choose between the PCA method (Primary Component Analysis, M. Arigovindan et al., 2005, IEEE Transactions on Image Processing 14. nr. 4 p.450ff) which is best suited for constrained iterative and fast iterative method and the Strata method (Myneni and Preza, Frontiers in Optics 2009, Optical Society of America, paper CThC4.), which is best for regularized inverse filter and Richardson Lucy iterative deconvolution.
Distance to cover slip	Displays the distance of the acquired structure from the side of the cover slip facing the embedding medium. Half the height of the Z-stack is assumed as the initial value for the distance from the cover slip. The value can be corrected if this distance is known. If possible, this distance should be measured. Note: Use **Ortho View** and the **Distance Measurement** option to define the distance of the sample to the coverslip. It is also important to estimate the position of the glass/embedding medium interface as precise as possible. If the z-stack extends into the coverslip, the determined range of the stack which reaches into the glass should be entered as a negative value. Example: Z-stack is 26 µm thick, glass/

Parameter	Description
	medium interface is positioned at 9 µm distance from the first plane of the stack. Resulting value for Distance to cover slip: - 9.0 µm. 
Cover slip type (Thickness and type)	Commercially available cover slips are divided into different groups depending on their thickness (0, 1, 1.5 and 2), which you can select from the dropdown list. Cover slips of the 1.5 type have an average thickness of 170 µm. In some cases, however, the actual values can vary greatly depending on the manufacturer. For best results the use of cover slips with a guaranteed thickness of 170 µm is recommended. Values that deviate from this can be entered directly in the **input field**.
Cover slip ref. index	Select the material that the cover slip is made of from the dropdown list. The corresponding refractive index is displayed in the input field next to it.
Working distance	Displays the working distance of the objective (i.e. the distance between the front lens and the side of the cover slip facing the objective). The working distance of the objective is determined automatically from the objective information, provided that the objective was selected correctly in the MTB 2011 Configuration program. You can, however, also enter the value manually.
Override	Only active if the **Enable correction** checkbox is activated. To reset the values, click on the **Reset** button.

12.7.3.2.4 Channel specific section

In this section you will find all settings that are channel-specific. This means that they may be configured differently for each channel.

Parameter	Description
Use external PSF checkbox	**Activated:** Uses an external measured PSF. You'll find an additional input window under **Image Parameters | Input** where you can choose the external PSF file. The software will check if the PSFs microscope parameters match with the input image. Deviations (10nm deviation in wavelength will be accepted) will make the software use a theoretical PSF.
Attach to input button	If an external PSF was selected you can attach the file to the input image. The saved input image will then contain the correct measured PSF. Usage of a theoretical PSF is possible as well for such an image. Just deactivate the **Use external PSF** checkbox.
Illumination display field	Displays the excitation wavelength for the channel dye [in nm] by using the peak value of the emission spectrum. The color field corresponds to the wavelength (as far as possible).
Detection display field	Displays the peak value of the emission wavelength for the channel dye. The color corresponds to the wavelength (as far as possible).
Sampling lateral display field	Depends on the geometric pixel scaling in the X/Y direction and displays the extent of the oversampling according to the Nyquist criterion. The value should be close to 2 or greater in order to achieve good results during DCV. As, in the case of widefield microscopes, this value is generally determined by the objective, the camera adapter used and the camera itself, it can only be influenced by the use of an Optovar. With confocal systems, the zoom can be set to match this criterion.
Sampling axial display field	Depends on the geometric pixel scaling in the Z direction and displays the extent of the oversampling according to the Nyquist criterion. The value should be at least 2 or greater in order to achieve good results during DCV. This value is determined by the increment of the focus drive during acquisition of Z-stacks and can therefore be changed easily.
Pinhole display field	Only available if a confocal microscope has been entered under the microscope parameters. Displays the size of the confocal pinhole in Airy units (AU).
NA Cond. display field	Only visible if the microscope is a **Conventional Microscope** and **Transmitted Light** has been selected as the illumination type. Displays the numerical aperture of the condenser with which transmitted-light acquisition was performed.

12.7.3.2.4.1 Microscope info section

Displays advanced microscope information that influences the form of the PSF in a channel-dependent way:

Parameter	Description
Illumination drop-down list	Here you can select the illumination method with which the data set has been acquired. In the event that a **Conventional Microscope** has been entered under the microscope parameters, the following options are available here: **Epifluorescence**, **Multiphoton Excitation** and **Transmitted Light**. In the case of confocal microscopes, **Epifluorescence** is the only option.
Image Formation	Displays whether the imaging was incoherent (**Conventional Microscope**) or coherent (**Laser Scanning Microscope**).
Lateral Resolution	Displays the lateral resolution of the calculated PSF.
Axial FWHM	Displays the FWHM (Full Width Half Maximum) as a measure of the axial resolution of the PSF.

12.7.3.2.4.2 PSF view section

This tool shows you the PSF that is calculated for a channel based on the current settings. If you select the **Auto Update** checkbox, all changes made to the PSF parameters are applied immediately to the PSF view. This makes it possible to check quickly whether the settings made meet your expectations.

1. To extract the PSF from the image, right click and select **PSF snapshot**.

The result is a new PSF document opened in the center screen area.

12.7.4 Creating a PSF - With Wizard and Without

The PSF Wizard combines two steps which are necessary for extracting experimental point spread functions (PSF) from Z-Stacks of subresolution fluorescent beads:

- A bead averaging step finds individual beads, presents them for inspection, allows you to select the ones you like and then creates an averaged combination of all selected beads. This stack shows a single bead which is, as a consequence of the averaging function, fairly free of noise.
- The averaged bead stack is then run through the **Create PSF** function which removes background and residual noise, correctly scales the PSF and also converts the stack into a 32-bit floating point format which is better suitable for the mathematical procedures used in deconvolution.

Prerequisite

✓ You have acquired a Z-Stack image. For more information, see *Measuring the PSF using subresolution beads [▸ 99]*.

✓ The use of the PSF wizard is activated.

1. Select the path to open the **Parameters** dialog box.
   → The functions **Use wizard** and **Bad Pixel Correction** are activated by default.
2. On the **Processing** tab, press the **Apply** button.
   → The PSF wizard opens and guides you throught the creation of the PSF.

→ If the **Use wizard** checkbox is not activated, the function shows the parameters for **Bead averaging**. Note that these parameters are only available in **Show all** mode. We recommend using the PSF wizard. The result of the wizard is a PSF file which you use in deconvolution for images acquired under the same conditions.

This method determines the position of fluorescent beads in a Z-stack image. If these beads are too close to one another they are excluded from the calculation. Beads which are far enough apart from one another are combined into a single bead, from which it is then possible to calculate a PSF using the **Create PSF** function (Processing / Utilities).

Description of the algorithm

This function consists of a series of steps before and after processing. The aim is to find beads that are far enough apart from one another. The processing steps are as follows:

- Select input image
- Image smoothing
- Segmentation
- Alignment of the center of the found beads
- Averaging of the beads

Parameters

- **Bead proximity**: Defines the distance between two neighboring beads in µm. A bead is excluded from the averaging if the distance to a neighboring bead is greater than the minimum distance set. A smaller value leads to the detection off fewer beads, albeit ones that are further apart, while a larger value leads to the detection of more beads, but with the risk that beads will partially overlap. Range between 2.1 and 20 µm.
- **Detection sensitivity**: determines the sensitivity with which beads are being detected. Range is between -5 to 5. Smaller Values lower the sensitivity excluding beads with weaker staining and lower signal to noise ratio, higher values include also beads with weaker staining.
- **Average Beads**: If activated, an image of an individual, averaged bead is produced. If deactivated, an image is produced in which each found bead is centered, but saved in the R dimension. A slider for Rotation (R dimension) appears on the **Dimensions** tab.

Bad Pixel Correction

This parameter employs a fully automatic detection and removal of spurious or hot pixels (also known as stuck pixels) in an image stack which might interfere with the PSF extraction procedure. It is based on the analysis of the gray level variance in the neighborhood of each pixel in the image. It is usually recommended to leave this parameter active.

12.7.5 Image types suitable for deconvolution

Most types of microscope images could in principle be deconvolved. However, there are practical limitations, for example the image file sizes might be too large or imaging conditions might be dominated by effects other than blurring by the point spread function. If, for example, a sample has strong light scattering properties or if light is strongly absorbed by the sample, deconvolution becomes difficult or impossible.

Deconvolution works both in 2D as in 3D. The PSF is very small in 2D, so the improvements of deconvolving 2D images are usually not very significant. Its full power deconvolution can show when processing 3D image stacks which have been acquired according to the following general rules:

- Acquisition of images with enough pixel resolution by choosing objectives with numerical apertures >0.5 and using camera resolutions with small enough pixel sizes as recommended by the Nyquist criterion.
- Acquisition of Z-stacks with distance between individual planes not larger than recommended by the Nyquist criterion (2 fold oversampling of the theoretically resolvable information, **Optimal** button in the Z-stack tool).
- Acquisition of enough planes above and below the structure of interest. As a rule, acquiring about half the axial PSF size above and below is enough to also get restoration of the structures at the top and bottom of the structure of interest.
- Avoiding saturation of the detector.
- Choosing imaging conditions to avoid sample bleaching.
- Avoiding spherical aberrations by choosing objectives, which use a immersion medium with a refractive index as close as possible to the mounting medium of the sample (for example using water immersion objectives for cell cultures in aqueous medium).
- Choosing sample media with low background fluorescence (for example phenol red free culture media).

ZEN deconvolution is suitable for images from many different microscope types. The following list of image types have been tested and are supported by ZEN deconvolution:

Imaging modality	Suitability for Deconvolution	Comment
Widefield fluorescence	+++	Ideally choose objectives with a numerical aperture >0.5.
LSM confocal imaging detecting	+++	Prerequisite is to have chosen a dye with the correct excitation and emission wavelengths before acquisition.
LSM Lambda and Online Fingerprinting imaging modes detecting fluorescence	++	Exact excitation and emission wavelengths are missing, need to be added on the PSF page before attempting deconvolution.
2-Photon (NLO) imaging using NDD (Non-descanned detectors)	+++	Excitation wavelength > emission wavelength.
ApoTome fluorescence	+++	Only ApoTome raw images should be deconvolved.
Spinning disk confocal	+	No spinning disk specific PSF model available, still can get good results when processing with a PSF according to standard confocal conditions (~1.2 airy units), or choose measured PSF.

Imaging modality	Suitability for Deconvolution	Comment
Lightsheet	-	Only single view deconvolution supported, image sizes might be challenging, best results for high NA objectives.
Airyscan	-	Airyscan raw data deconvolution is currently not supported, deconvolving already processed images is not recommended.
Elyra	-	Currently not supported.
Bright field transmitted light images	-	Not supported.
Axio Scan.Z1	+	Can be used for fluorescence stacks, however, frequently file sizes are prohibitive. Also, ideally JPEG-XR compression should not be used.
Celldiscoverer 7	+++	Very well suited due to objectives specialized for life cell imaging; recommended use of **Direct Processing** module to improve the workflow.

12.7.6 Deconvolution Methods in ZEN

Method (common name)	Reference	Settings	Comments
Nearest Neighbor	K. Castleman, “Digital image processing”, Prentice Hall 1997	Algorithm (default): **Nearest Neighbor**	Ad-hoc “2D de-blurring algorithm” focuses on subtraction of out of focus blur.
Regularized Inverse also known as: Linear Least Squares	For zero order g-difference: Schaefer et al. (2001)	Algorithm (default): **Regularized Inverse Filter** Advanced settings | Regularization: **Zero order**	Uses difference of observation and estimate as regularization term.
Regularized Inverse also known as: Linear Least Squares	For first order regularization, or *Good's roughness*: Verveer et al. (1997)	Algorithm: **Regularized Inverse** Advanced settings | Regularization: **First order**	Uses *Good's roughness* first derivative of estimate as regularization term.

Method (common name)	Reference	Settings	Comments
Regularized Inverse also known as: Linear Least Squares In conjunction with structured illumination microscopy (ApoTome)	Schaefer et al. (2006) Schaefer et al. (tbs)	Algorithm: **Regularized Inverse** Advanced settings | Regularization: **Zero / First order**	Patented method for maximum exploitation of ApoTome raw images
Fast Iterative Also known as: Meinel Algorithm Gold Meinel	Meinel (1986)	Algorithm (default): **Fast Iterative** Advanced settings | Likelihood: **Poisson (Meinel)**	Classic, non-regularized Meinel algorithm.
Fast Iterative Meinel Algorithm + Regularization:	Meinel (1986), For zero order g-difference: Schaefer et al. (2001)	Algorithm: **Fast Iterative** Advanced settings: - Likelihood: **Poisson (Meinel)** - Regularization: **Zero order**	Regularized Meinel algorithm using g-difference (difference of observation and estimate) term.
Fast Iterative Meinel Algorithm + Optimization	Meinel (1986), Biggs (1998)	Algorithm: **Fast Iterative** Advanced settings: - Likelihood: **Poisson (Meinel)** - Regularization: **None / Zero order** - Optimization: **Numerical Gradient**	Meinel algorithm using a numerical gradient estimator as proposed by D. Biggs.
Fast Iterative Also known as: Richardson Lucy (RL) Algorithm	Richardson (1972) Lucy (1974)	Algorithm: **Fast Iterative** Advanced settings | Likelihood: **Poisson (Richardson, Lucy)**	Classic, original non-regularized Richardson Lucy algorithm. May need many more iterations than any other algorithm.
Fast Iterative Also known as:	Richardson (1972) Lucy (1974) Biggs (1998)	Algorithm: **Fast Iterative** Advanced settings:	Classic, original non-regularized Richardson Lucy algorithm. Improved rate of

Method (common name)	Reference	Settings	Comments
Richardson Lucy Algorithm + Optimization		- Likelihood: **Poisson (Richardson, Lucy)** - Optimization: **Numerical Gradient**	convergence. About a factor of 10 faster than RL using a numerical gradient estimator as proposed by D. Biggs.
Constrained Iterative	Verveer et al. (1997) Schaefer et al. (2001)	Algorithm (default): **Constrained Iterative** Advanced settings: - Likelihood: **Poisson** - Regularization: **Zero order**	Generic conjugate gradient restoration using squared estimate to impose positivity. Uses difference of observation and estimate as regularization term.
Constrained Iterative	Verveer et al. (1997) Schaefer et al. (2001)	Algorithm: **Constrained Iterative** Advanced settings: - Likelihood: **Poisson** - Regularization: **First order**	Generic conjugate gradient restoration using squared estimate to impose positivity. Uses *Good's roughness* derivative operator as regularization term.
Constrained Iterative	Tikhonov (1977) Verveer et al. (1997) Schaefer et al. (2001)	Algorithm: **Constrained Iterative** Advanced settings: - Likelihood: **Poisson** - Regularization: **Second orde**r	Generic conjugate gradient restoration using squared estimate to impose positivity. Uses *Tikhonov Miller Phillips* second derivative operator as regularization term.
Constrained Iterative	Verveer et al. (1997) Schaefer et al. (2001)	Algorithm: **Constrained Iterative** Advanced settings: - Likelihood: **Gauss** - Regularization: **Zero order**	Generic conjugate gradient restoration using squared estimate to impose positivity. Uses difference of observation and estimate as regularization term.

Method (common name)	Reference	Settings	Comments
Constrained Iterative	Verveer et al. (1997) Schaefer et al. (2001)	Algorithm: **Constrained Iterative** Advanced settings: - Likelihood: **Gauss** - Regularization: **First order**	Generic conjugate gradient restoration using squared estimate to impose positivity. Uses *Good's roughness* derivative operator as regularization term.
Constrained Iterative Also known as: ICTM Iterative Constrained Tikhonov Miller	van der Voort et al. (1995) Verveer et al. (1997) Schaefer et al. (2001)	Algorithm: **Constrained Iterative** Advanced settings: - Likelihood: **Gauss** - Regularization: **Second order**	Generic conjugate gradient restoration using squared estimate to impose positivity. Uses *Tikhonov Miller Phillips* second derivative operator as regularization term.
Constrained Iterative	Verveer et al. (1997) Schaefer et al. (2001)	Algorithm: **Constrained Iterative** Advanced settings: - Likelihood: **Poisson/Gauss** - Regularization: **0/1/2nd order** - Optimization: **Line search / Analytical**	Generic conjugate gradient restoration using squared estimate to impose positivity. **Default for optimization** is the fast analytical (Newton Raphson) method. Line search may be more accurate but is also much slower

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12.7.7 Table of Default Parameter for Deconvolution

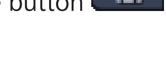
The following table lists the parameters which are used by default for widefield, confocal, light-sheet and ApoTome images.

Microscope Type	Widefield	Confocal	Lightsheet	ApoTome
General Deconvolution Parameter Defaults				
Normali- zation	Auto	Auto	Auto	Auto
Backgound Cor- rection	Off	Off	Off	Off
Flicker Correc- tion	Off	Off	Off	Off
Decay Correc- tion	Off	Off	Off	Off
Hot Pixel Cor- rection	Off	Off	Off	Off
Constrained Iterative Specific Defaults				
Strength (auto- matic, manual; range 0..10)	Auto	Auto	Manual (str 5)	Auto
Likelihood	Poisson	Poisson	Poisson	—
Regulari- zation	ZeroOrder	FirstOrder	ZeroOrder	—
Optimization	Analytical	LineSearch	Analytical	—
First Estimate	Input	Mean	Input	—
Maximum Num- ber Of Itera- tions	40	7	40	—
Auto Stop Per- centage	0.1	0.1	0.1	—
Fast Iterative Specific Defaults				
Method	Poisson / Meinel	Poisson / Richardson Lucy	Poisson / Meinel	—

Microscope Type	Widefield	Confocal	Lightsheet	ApoTome
Regulari- zation	None	None	None	—
Optimization	None	None	None	—
FirstEstimate	Input	Mean	Input	—
Maximum Num- ber Of Itera- tions	15	50	15	—
AutoStop Per- centage	0.1	0.1	0.1	—
Regularized Inverse Specific Defaults				
Regulari- zation	Zero Order	Zero Order	Zero Order	First Order

12.7.8 Creating Deconvolution Settings

You can create settings for Deconvolution which can be saved, exported, and imported.

1. Open the **Processing** tab and select the method **Deconvolution (adjustable)**.
2. In the **Parameters** window, activate **Show All** (if it is not already activated).
3. In the **Input** window, select the desired image for the Deconvolution.
   Note: If you use the settings for Direct Processing, use a test image acquired with the iden- tical experiment settings you will be using when running the experiment with Direct Pro- cessing.
4. Click on the context menu button and select **New** from the drop-down list.
5. Enter a name for your settings and press the *Enter* key or click on the save button .
6. Configure your settings in the **Deconvolution** or **PSF settings** tab.
   For more information, see the help for *Deconvolution tab [▸ 323]* and *PSF Settings tab [▸ 329]*.
7. Click on the context menu button and select **Save**.

You have now created and saved a Deconvolution setting. You can load this setting into the **Di- rect Processing** tool to use it for a Direct Processing experiment.

NOTICE

GPU

The setting also saves the status of the GPU. If you create the setting on a machine without a GPU, export it, and import the setting on machine with GPU, the GPU will not be used. There- fore, the processing can be considerably slow. In this case, we recommend to create the set- ting directly on the machine where the processing is executed.

Exception for Direct Processing:
If you set up your experiment and your setting on an acquisition PC without a GPU, the pro- cessing PC will ignore the status and use the GPU (if available).

See also

Using Direct Processing with advanced deconvolution settings [▸ 350]

12.8 Direct Processing

Direct Processing introduces functionalities directed to increase speed, usability, and reduce costs for processing. Firstly, with **Direct Processing** you can select a processing function, which is then directly executed as images are acquired. With this processing function, you save time by avoiding lengthy post processing steps. Several different functions are available for **Direct Processing**. You can also define a sequence of functions (in a so-called pipeline), which are then executed one after another.

Secondly, **Direct Processing** allows an acquisition computer to communicate with a second PC (processing computer) connected via a network connection. The acquisition computer instructs the processing computer to process images as they are being acquired. Before you can use **Direct Processing**, you need to connect the two computers. As there are numerous ways, how computers can be set up to become networked, we can only give some general advice here. Contact your local IT administration for help on how to configure the computers in accordance to the local infrastructure.

One way is to connect the computer controlling the microscope system to a second computer via a direct ethernet connection. If Network Discovery is switched on, Windows 10 will directly support such a point to point connection. Create a shared folder which can be accessed from both computers. Since no other network traffic will use this connection, the whole bandwidth will be available for saving data directly from the acquisition computer to a shared folder on the processing computer. This is the most efficient way as the acquired data do not have to be copied off the acquisition computer after the acquisition has finished.
Alternatively, it is also possible, to use a processing computer which is already integrated into an existing network. Depending on the network type and the kind of experiments being done, the bandwidth might not be sufficient to directly stream data to the processing computer. In such cases, to not limit the throughput of the acquisition, it is advisable to let the acquisition computer acquire data to a local drive and instruct the processing computer where to look for the acquired data.

For a communication via network, it is possible to link both computers with a discovery proxy. The discovery proxy is a service where you can register all available processing computers and your acquisition computer can then ask this service for the list of these available PCs. You can also link the PCs without such a discovery service, for which you then need the IP address and/ or network name of the processing computer.

Note: It is possible to set up **Direct Processing** to acquire and process images on one and the same workstation.

12.8.1 Direct Processing

This step-by-step-guide shows you how to perform Direct Processing.

- Before using the Direct Processing functionality, the acquisition and the processing computers need to be connected.
  For more information, see also *Connecting the acquisition computer with the processing computer [▶ 344]*.
- On the acquisition computer, settings need to be defined.
  For more information, see also *Direct Processing Tool on Acquisition Computer [▶ 351]*.
- On the acquisition computer, settings in the **Auto Save** tool need to be defined.
  For more information, see also *Defining settings in the Auto Save tool [▶ 346]*.
- On the processing computer, the receiving needs to be activated.
  For more information, see also *Direct Processing Tool on Processing Computer [▶ 354]*.

- Set up and run an experiment with Direct Processing.
  For detailed information, see also *Using Direct Processing [▸ 346]*.

See also

Using Direct Processing with advanced deconvolution settings [▸ 350]
Using Direct Processing with Airyscan [▸ 347]
Using Direct Processing with Deconvolution [▸ 349]

12.8.2 Connecting the acquisition computer with the processing computer

Before you can apply deconvolution on a processing computer, you need to connect the two computers. For general information, see the *Direct Processing [▸ 343]*. For the communication via network with or without a discovery proxy, please refer to the chapters *Connecting the computers without discovery proxy [▸ 344]* or *Connecting the computers with discovery proxy [▸ 345]* respectively.

12.8.2.1 Connecting the computers without discovery proxy

On the Processing PC

Prerequisite ✓ You are on the processing computer.

1. Open **Tools > Options > Direct Processing**.
2. Open the **Setup Processing PC** section and make sure that **Announcement to Discovery Proxy** is deactivated.
3. Note down the name and/or the IP address and port of the processing PC. You can find the IP and port under **IP Address (this PC)** and the computer name if you activate **Send Hardware Information**. Alternatively, ask your IT department for information on how to find the IP address or name of the computer.
   Note: In some networks the computers might get assigned a new IP address over time (e.g. each day), so using the computer name for establishing communication would be advisable.
4. If you want to display information about the processing computer on your acquisition computer, enter the information in the text box **PC Description**. If you want to have the hardware information, activate **Send Hardware Information**, and for displaying statistics about the average job time, activate **Send Processing Statistics**.
5. Click on **OK** to close the **Tools > Options** dialog.
6. On the **Applications** tab, in the **Direct Processing** tool, click on **Start Receiving**.

Everything is now set up on the processing computer.

On the Acquisition PC

Prerequisite ✓ You are on the acquisition computer.

1. Open **Tools > Options > Direct Processing**.
2. Open the **Setup Processing PC** section and make sure that **Announcement to Discovery Proxy** is deactivated.
3. Click on **OK** to close the **Tools > Options** dialog.
4. On the **Acquisition** tab, activate **Direct Processing**. This automatically activates **Auto Save** as well.
5. In the **Direct Processing** tool, click on the **Edit Connection** button.
   → The **Connected Processing PCs** dialog opens.
6. In the text fields, enter the name or the IP address and port of the processing computer.

7. Click on **Add**.
   → The processing computer is now added to the list.
8. Select your processing computer in the list and click on **Connect**. Alternatively, activate **Automatically Select Processing PC** and the processing PC is automatically selected for each experiment based on the available PCs and their queue length (the PC with the shortest queue is selected).
   → You are now connected to the processing computer.
9. Click on **Close** to exit the dialog.

Both computers are now connected and Direct Processing is set up. You can now configure and run your experiment.

See also

- Direct Processing [▹ 343]
- Using Direct Processing with Deconvolution [▹ 349]
- Using Direct Processing with advanced deconvolution settings [▹ 350]
- Using Direct Processing with Airyscan [▹ 347]

12.8.2.2 Connecting the computers with discovery proxy

On the Processing PC

Prerequisite ✓ You are on the processing computer.

1. Open **Tools > Options > Direct Processing**.
2. Open the **Setup Processing PC** section and activate the checkbox **Announcement to Discovery Proxy**.
3. Under **IP Address (Discovery Proxy)**, enter the IP address and port of the discovery proxy.
4. If you want to display information about the processing computer on your acquisition computer, enter the information in the text box **PC Description**. If you want to have the hardware information, activate **Send Hardware Information**, and for displaying statistics about the average job time, activate **Send Processing Statistics**.
5. Click **OK** to close the **Tools > Options** dialog and save the settings.
6. On the **Applications** tab, in the **Direct Processing** tool, click on **Start Receiving**.

Everything is now set up on the processing computer.

On the Acquisition PC

Prerequisite ✓ You are on the acquisition computer.

1. Open **Tools > Options > Direct Processing**.
2. Open the **Setup Acquisition PC** section and activate the checkbox **Find from Discovery Proxy**.
3. Under **IP Address (Discovery Proxy)**, enter the address of the Discovery Proxy. Alternatively, this can also be done or changed in the **Connected Processing PCs** dialog later.
4. Click on **OK** to close the **Tools > Options** dialog.
5. On the **Acquisition** tab, activate **Direct Processing**. This automatically activates **Auto Save** as well.
6. In the **Direct Processing** tool, click on the **Edit Connection** button.
   → The **Connected Processing PCs** dialog opens.

7. Select your processing computer in the list and click on **Connect**. Alternatively, activate **Automatically Select Processing PC** and the processing PC is automatically selected for each experiment based on the available PCs and their queue length (the PC with the shortest queue is selected).
   → You are now connected to the processing computer.
8. Click on **Close** to exit the dialog.

Both computers are now connected and Direct Processing is set up. You can now configure and run your experiment.

See also

- Direct Processing [▸ 343]
- Using Direct Processing with Deconvolution [▸ 349]
- Using Direct Processing with advanced deconvolution settings [▸ 350]
- Using Direct Processing with Airyscan [▸ 347]

12.8.3 Setting up your PC as discovery proxy

If you want to set up and use your computer as discovery proxy, take the following steps:

1. Open **Tools > Options > Direct Processing > General Options**.
2. For **Communication Mechanism**, select **Network Based (WCF)**.
3. Open the **Setup Discovery Proxy Server** section and click on **Start**.
4. Click on **OK** to close the **Tools > Options** dialog.

This computer is now set up and used as discovery proxy.

12.8.4 Defining settings in the Auto Save tool

When using the Direct Processing functionality, perform the following steps on the acquisition computer to define the settings in the **Auto Save** tool.

Prerequisite ✓ **Acquisition tab > Auto Save** tool is open.

1. In the **Folder** field, specify the directory, where acquired images will be stored. Under normal circumstances, this is automatically the folder, which has been specified as the communication folder. However, make sure that it is a shared folder which can be accessed from both computers.
2. If you want images to automatically be stored in a new subfolder named with the current date, activate the **Automatic Sub-Folder** option.
3. In the **Name** field, specify the file base name for the acquired images.
4. Activate the **Close CZI Image After Acquisition** option to release them from the acquisition computers memory.

On your acquisition computer, you have defined the settings in the **Auto Save** tool.

12.8.5 Using Direct Processing

Prerequisite

✓ You have connected acquisition and processing computer. For more information, see *Connecting the acquisition computer with the processing computer [▸ 344]*.

✓ To ensure that the processing computer reads incoming files and starts the processing, on the **Applications** tab **> Direct Processing** tool, you have clicked the **Start Receiving** button.

✓ On the **Acquisition** tab, you have set up your experiment for image acquisition.

- ✓ On the **Acquisition** tab, **Direct Processing** is activated. This activates the **Auto Save** tool as well.
- ✓ Depending on your settings, you have defined the folder where the acquired images are stored in the **Direct Processing** or the **Auto Save** tool. For more information, see *Auto Save Tool [▸ 766]*. Use a folder to which the processing computer has access.

1. On the **Acquisition** tab, open the **Direct Processing** tool.
2. From the **Processing Function** drop-down list, select the processing function you want to use.
   → The parameters of the function are displayed and the name of the function is displayed in the pipeline container.
3. Set all the parameters of the function for your experiment. For detailed information about the parameters refer to the descriptions of the individual image processing function.
4. To add another function or several other ones, click on **Add Function**.
   → A new container is added in the pipeline.
5. Select the next pipeline container, select a processing function from the dropdown, and set the parameters for each function.
   → You have added and set up a sequence of processing functions.
6. Click on **Start Experiment** to run the experiment. Note: You can pause the processing. If you stop the experiment, requests that have been sent earlier by the acquisition computer are not processed. However, already processed images will be retained.
   → The images are stored in the folder you have defined in the **Auto Save** or **Direct Processing** tool. When you abort the acquisition, the remote processing will not take place. In case you have set up several processing functions, only the acquired image and the final output image are stored.
   → The processing computer reads incoming files and starts the processing. The path to the selected folder, the currently processed image as well as the images to be processed are displayed in the **Direct Processing** tool. The processed image is saved to the same folder specified in the **Direct Processing** tool. If the image name already exists in this folder, the new file is saved under a new name <oldName>-02.czi.
7. To cancel the processing on the processing computer, on the **Applications** tab, in the **Direct Processing** tool, click on the **Cancel Processing** button.

Once processing is finished, you are notified on the acquisition PC and can open and view the acquired image as well as the processed image. This should be done on the processing computer, so that you can immediately start a new experiment on the acquisition computer. However, you can also automatically open the processed image on the acquisition PC with the respective setting on the *Direct Processing Tool on Acquisition Computer [▸ 351]*.

Information about Direct Processing (e.g. the duration) is available on the **Info** tab of the processed image.

See also

- Using Direct Processing with Airyscan [▸ 347]
- Using Direct Processing with Deconvolution [▸ 349]
- Using Direct Processing with advanced deconvolution settings [▸ 350]

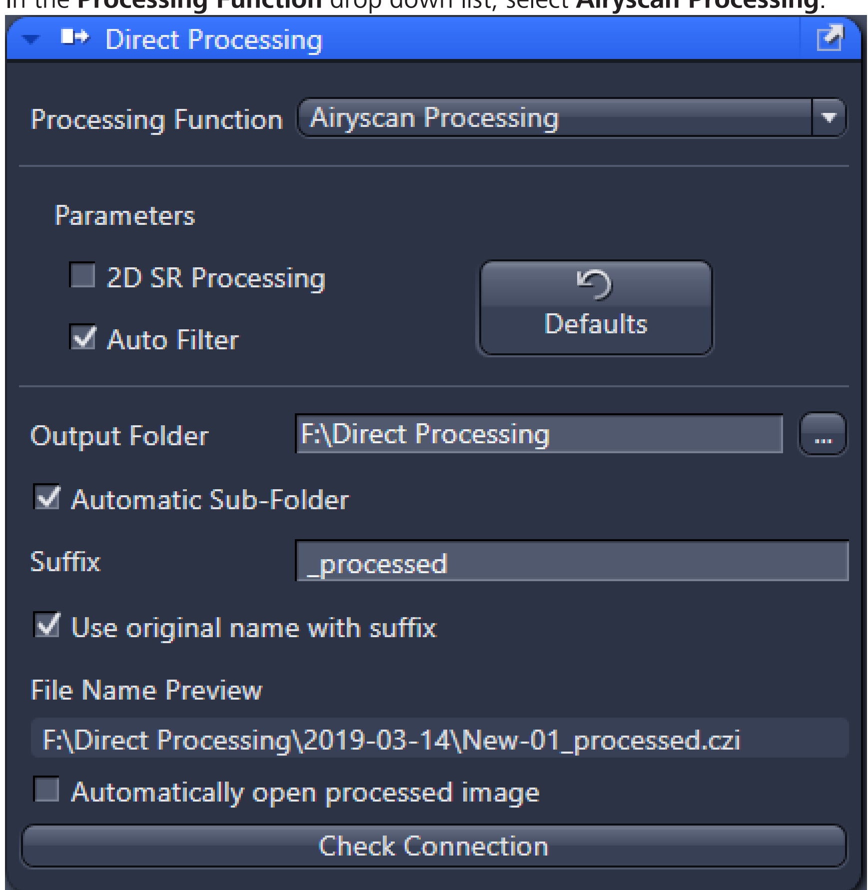
12.8.6 Using Direct Processing with Airyscan

Prerequisite

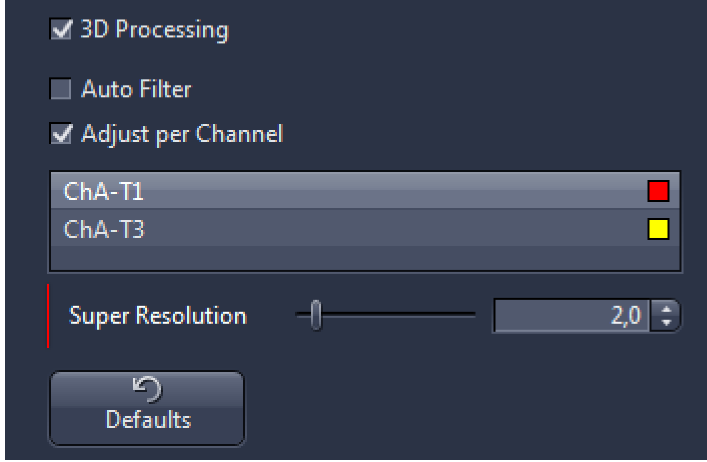
- ✓ This function is only available, if an Airyscan detector is available.
- ✓ You have connected acquisition and processing computer. For more information, see *Connecting the acquisition computer with the processing computer [▸ 344]*.
- ✓ You have defined the settings on the acquisition computer. For more information, see *Direct Processing Tool on Acquisition Computer [▸ 351]*.

- ✓ To ensure that the processing computer reads incoming files and starts the processing, on the **Applications tab > Direct Processing** tool you have clicked the **Start Receiving** button.
- ✓ On the **Acquisition** tab, you have set up your experiment for image acquisition.
- ✓ On the **Acquisition tab**, **Direct Processing** is activated. This activates the **Auto Save** tool as well.
- ✓ Depending on your settings, you have defined the folder where the acquired images are stored in the **Direct Processing** or the **Auto Save** tool. For more information, see *Auto Save Tool [▸ 766]*. Use a folder to which the processing computer has access.

1. In the **Processing Function** drop down list, select **Airyscan Processing**.



Note: If you uncheck the **Auto Filter** checkbox and activate **Adjust per Channel**, you can set the **Super Resolution** parameter in a channel specific manner.



2. Select the desired settings for **Airyscan Processing**. For details how to use of this function, see *Airyscan Processing [▸ 177]*. Ideally, you have already checked the best parameters beforehand, using a sample image acquired under the same conditions as set up for the experiment.
3. Click on **Start Experiment** to run the experiment.
   Note: You can pause the processing. If you stop the experiment, requests that have been sent earlier by the acquisition computer are not processed. However, already processed images will be retained.
   → The images are stored in the folder you have defined in the **Auto Save** or **Direct Processing** tool. When you abort the acquisition, the remote Airyscan processing will not take place.
   → The processing computer reads incoming files and starts the Airyscan processing. The path to the selected folder, the currently processed image as well as the images to be processed are displayed in the **Direct Processing** tool. The processed image is saved to the same folder specified in the **Direct Processing** tool. If the image name already exists in this folder, the new file is saved under a new name <oldName>-02.czi.
4. To cancel the processing, click on the **Cancel Processing** button.

Once processing is finished, you are notified on the acquisition PC and can open and view the acquired image as well as the processed images. This should be done on the processing computer so that you immediately can start a new experiment on the acquisition computer. However, you can also automatically open the processed image on the acquisition PC with the respective setting on the *Direct Processing Tool on Acquisition Computer [▸ 351]*.

When you open the image in the **Image View**, information about the executed Airyscan processing is available on the **Info** tab. Additionally, general information about Direct Processing (e.g. the duration) is also available on the **Info** tab of the processed image.

12.8.7 Using Direct Processing with Deconvolution

Specify, how images should be processed during acquisition.

Prerequisite

- ✓ You have connected acquisition and processing computer. For more information, see *Connecting the acquisition computer with the processing computer [▸ 344]*.
- ✓ You have defined the settings on the acquisition computer. For more information, see *Direct Processing Tool on Acquisition Computer [▸ 351]*.
- ✓ To ensure that the processing computer reads incoming files and starts the processing, on the **Applications** tab **> Direct Processing** tool you have clicked the **Start Receiving** button.
- ✓ On the **Acquisition** tab, you have set up your experiment for image acquisition.
- ✓ On the **Acquisition** tab, **Direct Processing** is activated. This activates the **Auto Save** tool as well.
- ✓ Depending on your settings, you have defined the folder where the acquired images are stored in the **Direct Processing** or the **Auto Save** tool. For more information, see *Auto Save Tool [▸ 766]*. Use a folder to which the processing computer has access.

1. Select **Acquisition** tab **> Direct Processing** tool and select the processing function **Deconvolution** from the drop-down list. For details about Deconvolution operation and which type of images are suitable for deconvolution, see *Performing Deconvolution Using Default Values [▸ 91]*.
2. Select a deconvolution method. We recommend **Excellent, slow (Constraint Iterative)**.
3. Set up the experiment. For optimal processing efficiency, select the **Full Z-Stack per channel** option. This way, the processing can start as soon as a channel-Z-Stack has been completed.
4. Click on **Start Experiment** to run the experiment. Note: You can pause the processing. If you stop the experiment, requests that have been sent earlier by the acquisition computer are not processed. However, already processed images will be retained.

→ The images are stored in the folder you have defined in the **Auto Save** or **Direct Processing** tool. When you abort the acquisition, the remote deconvolution will not take place.

→ The processing computer reads incoming files and starts the deconvolution processing. The path to the selected folder, the currently processed image as well as the images to be processed are displayed in the **Direct Processing** tool. The processed image is saved to the same folder specified in the **Direct Processing** tool. If the image name already exists in this folder, the new file is saved under a new name <oldName>-02.czi.

5. To cancel the processing on the processing computer, on the **Applications** tab, in the **Direct Processing** tool, click on the **Cancel Processing** button.

Once processing is finished, you are notified on the acquisition PC and can open and view the acquired image as well as the processed images. This should be done on the processing computer, so that you immediately can start a new experiment on the acquisition computer. However, you can also automatically open the processed image on the acquisition PC with the respective setting on the *Direct Processing Tool on Acquisition Computer [▸ 351]*.

When you open the image, in the **Image** View, on the **Info** tab, information about the executed deconvolution is available. When Deconvolution is done through Direct Processing, the info about Deconvolution parameters shows the suffix **online** and the **Convergence History** graph. Additionally, general information about Direct Processing (e.g. the duration) is also available on the **Info** tab of the processed image.

12.8.8 Using Direct Processing with advanced deconvolution settings

Prerequisite

- ✓ You have connected acquisition and processing computer. For more information, see *Connecting the acquisition computer with the processing computer [▸ 344]*.
- ✓ You have defined the settings on the acquisition computer. For more information, see *Direct Processing Tool on Acquisition Computer [▸ 351]*.
- ✓ To ensure that the processing computer reads incoming files and starts the processing, on the **Applications** tab **> Direct Processing** tool you have clicked the **Start Receiving** button.
- ✓ On the **Acquisition** tab, you have set up your experiment for image acquisition.
- ✓ On the **Acquisition** tab, **Direct Processing** is activated. This activates the **Auto Save** tool as well.
- ✓ Depending on your settings, you have defined the folder where the acquired images are stored in the **Direct Processing** or the **Auto Save** tool. For more information, see *Auto Save Tool [▸ 766]*. Use a folder to which the processing computer has access.
- ✓ You have advanced settings for Deconvolution. For more information, see *Creating Deconvolution Settings [▸ 342]*.

1. On the **Acquisition** tab, open the **Direct Processing** tool.
2. Select the processing function **Deconvolution** from the drop-down list.
3. Activate the checkbox **Use advanced Settings**.
4. In the drop-down list, select your advanced settings for Deconvolution.
   Note: Currently Direct Processing supports settings configured in the **Deconvolution** tab of the image processing function **Deconvolution (adjustable)** and some settings of the **PSF** tab.
5. Set up the experiment. For optimal processing efficiency, select the **Full Z-Stack per channel** option. This way, the processing can start as soon as a channel-Z-Stack has been completed.
6. Click on **Start Experiment** to run the experiment. Note: You can pause the processing. If you stop the experiment, requests that have been sent earlier by the acquisition computer are not processed. However, already processed images will be retained.

→ The images are stored in the folder you have defined in the **Auto Save** or **Direct Processing** tool. When you abort the acquisition, the remote Deconvolution will not take place.

→ The processing computer reads incoming files and starts the Deconvolution processing. The path to the selected folder, the currently processed image as well as the images to be processed are displayed in the **Direct Processing** tool. The processed image is saved to the same folder specified in the **Direct Processing** tool. If the image name already exists in this folder, the new file is saved under a new name <oldName>-02.czi.

7. To cancel the processing on the processing computer, on the **Applications** tab, in the **Direct Processing** tool, click on the **Cancel Processing** button.

Once processing is finished, you are notified on the acquisition PC and can open and view the acquired image as well as the processed images. This should be done on the processing computer, so that you immediately can start a new experiment on the acquisition computer. However, you can also automatically open the processed image on the acquisition PC with the respective setting on the *Direct Processing Tool on Acquisition Computer [▸ 351]*.

When you open the image, in the **Image** View, on the **Info** tab, information about the executed Deconvolution is available. When Deconvolution is done through Direct Processing, the info about Deconvolution parameters shows the suffix **online** and the **Convergence History** graph. Additionally, general information about Direct Processing (e.g. the duration) is also available on the **Info** tab of the processed image.

12.8.9 Functions & Reference

12.8.9.1 Direct Processing Tool on Acquisition Computer

Parameter	Description
Add Function	Adds another processing function to the pipeline.
Remove Function	Removes the currently selected processing function from the pipeline.
Pipeline	Displays a list of containers for possible processing functions. To set the function for the currently selected container, use the **Processing Function** dropdown list. The functions in this pipeline are executed one after the other in the sequence as given here. You can change the position of a function in the pipeline via drag & drop.
Processing Function	Selects the processing function you want to use for **Direct Processing** for the currently selected container of the pipeline.
Parameter section	In this section you have different parameters, depending on the selected **Processing Function**. For more information, see: ▪ *Airyscan Processing Parameters [▸ 353]* ▪ *ApoTome RAW Convert Parameters [▸ 179]* ▪ *Deconvolution Parameters [▸ 353]* ▪ *Denoise Parameters [▸ 116]* ▪ *Unsharp Mask Parameters [▸ 159]*
File Settings	Displays options to adjust setting for the output image(s).
- Output Folder	Displays the path where the processed image is saved. Make sure that a shared folder is selected where both the acquisition and the processing computers have access to.

Parameter	Description
- Use Output Folder from Auto-Save	**Activated:** Synchronizes the output folder for Direct Processing with the folder in the **Auto Save** tool. The **Output Folder** can only be changed by editing the **Folder** in the **Auto Save** tool respectively.
- Create Sub-folders Automatically	**Activated**: Creates sub-folders in the output folder. All processed images end up in a new folder named with the current date.
- Define File Naming	Defines the filename of the processed image. Default: **processed.czi**.
- Use Original Name with Suffix	**Activated:** Uses the name of the file as it was acquired and adds the defined file naming as a suffix.
- File Name Preview	Displays a preview of the name of the processed acquisition image.
Processed Image	Selects if/how the processed image should be displayed here on the acquisition PC.
- Do Not Open Automatically	The processed image is not opened automatically.
- Display in Split View	The processed image is displayed in a splitter document together with the acquired image as soon as the first image data have been processed.
- Only Open Final Image	The processed image is displayed after processing is completed.
Connected Processing PC	Displays the status of your connection to a processing PC (e.g. to which you are connected, how the progress of your job is). If you are not connected to a PC, you can edit your connections here.
- Edit Connection	Opens the *Direct Processing PCs Dialog [▸ 355]* which lists all the connected PCs that are ready to receive remote processing requests. In case of file based communication, it checks if there is a processing PC listening to the communication path. A message informs you about the result.

For more information, see:

- *Using Direct Processing [▸ 346]*
- *Using Direct Processing with Airyscan [▸ 347]*
- *Using Direct Processing with Deconvolution [▸ 349]*
- *Using Direct Processing with advanced deconvolution settings [▸ 350]*

12.8.9.1.1 Airyscan Processing Parameters

This set of parameters is only visible if **Airyscan Processing** is selected as **Processing Function**. For the information, you can also see *Airyscan Processing [▸ 177]*.

Parameter	Description
3D Processing	Only available for images with 5 or more z-positions. If activated, this option improves the resolution in axial and lateral direction. The data set needs to have at least 5 z-sections acquired with an optimal step size. 3D Processing is slower than 2D Processing. For 3D Processing, the whole z-stack (single channel and time point) needs to fit into the physical memory.
2D SR Processing	Only available for 2D images. Enhances the 2D resolution. Note this only results in increased super resolution when images are acquired with optimal settings and sufficient signal.
Auto Filter	If activated, a suitable **Super Resolution** parameter for the Airyscan processing is automatically determined for the selected data set. To manually adjust the **Super Resolution** parameter, deactivate the checkbox.
Strength	Use this option for an increased (high) or decreased (low) strength of the automatically assigned filter value. This is especially useful for 3D processing, as the 2D preview of the processing filter value in the Airyscan viewer does not allow to conclude the result after a 3D data processing. The increment of this parameter is ± 0.4 compared to the standard auto Airyscan processing. This setting is not available when manual processing strength is selected.
Adjust per Channel	Only visible, if **Auto Filter** is deactivated. Only available for images with two or more Airyscan channels. If activated, you can manually set channel specific Airyscan processing parameters.
Super Resolution	Only available if **Auto Filter** is deactivated. Manually adjusts the **Super Resolution** parameter. **Note:** High strength might look attractive at some images, Z planes or color channels, but other filtering artefacts might occur which appear like small rings in the image. Also the results will become very sharp, but grainy. So carefully check your image data in order to avoid such artefacts.

12.8.9.1.2 Deconvolution Parameters

This set of parameters is only visible if **Deconvolution** is selected as **Processing Function**.

Parameter	Description
Use advanced settings	**Activated**: Enables the drop-down menu to load an advanced Deconvolution setting.

Parameter	Description
Load Deconvolution Setting created in the Deconvolution function	Only available if **Use advanced settings** is activated. Select advanced settings which were created in the image processing function **Deconvolution (adjustable)**. For more information, see *Creating Deconvolution Settings [342]*. **Note**: Currently Direct Processing only supports settings configured in the **Deconvolution** tab of the image processing function, the settings on the **PSF** tab such as spherical aberrations correction cannot be used in Direct Processing.
- Simple, very fast (Nearest Neighbor)	Only available if **Use advanced settings** is deactivated. If **Deconvolution** is selected, the parameters are the same as for **Deconvolution (defaults)** except that Normalization will be set to **Clip** in case of remote processing (normally it is **Automatic**). This is because only with **Clip** the output images show brightness values which allow quantitative comparisons. For **Direct Processing**, you cannot change any other values. We recommend **Excellent, slow (Constraint Iterative)**. For more information, see *Deconvolution (defaults) [323]*, for advanced Deconvolution see *Deconvolution (adjustable) [323]*.
- Better, fast (Regularized Inverse Filter)	
- Good, medium speed (Fast Iterative)	
- Excellent, slow (Constraint Iterative)	

12.8.9.2 Direct Processing Tool on Processing Computer

Parameter	Description
Start / Stop Receiving	Starts or stops the reception of processing requests. The processing computer waits for processing requests from the acquisition computer. **Note:** If you click on **Stop Receiving** while an experiment is running, the experiment is continued and processed. Only after finishing the currently running experiment, a new experiment is processed.
Listening on	Shows the path where the computer is listening for processing requests.
Current request	Opens a dialog which shows the **Listening** directory. The current request field shows, which image is currently being processed. A progress bar informs you, how close you are to completing the currently processed image.
Items in the queue	Displays the number of images to be processed. Note that due to the integrative nature of the CZI images, individual scenes will not show up as individual steps in the queue. Only when separated CZI documents are being produced, will the queue show a count >0.
Cancel Processing	Cancels the processing of the images in the output folder.

For more information, see *Direct Processing [343]*.

12.8.9.3 Direct Processing PCs Dialog

Parameter	Description
Use Discovery Proxy	**Activated:** Uses a Discovery Proxy server for the communication between the computers. This control is synchronized with the respective options in the **Tools > Options > Direct Processing** dialog.
– Host Name	Only available, if **Use Discovery Proxy** is activated. Displays and edits the name/IP address of the discovery proxy.
– OK	Uses the defined Discovery Proxy.
Automatically Select Processing PC	**Activated:** Automatically selects a processing PC for each experiment based on the available PCs and their queue length (the PC with the shortest queue is selected).
Available Processing PCs list	Displays a list with all the available processing PCs. It provides the name, an overview of how many jobs are currently in the **Queue**. Your current PC is automatically listed, if you have clicked **Start Receiving** in the **Direct Processing** tool on the **Applications** tab.
– Connect	Connects to the respective processing PC.
– Delete	Only available for PCs that are added as **Custom Processing PC**. Deletes the custom PC from the list.
Custom Processing PC	Defines a custom PC for processing.
– Host Name	Sets the name/IP address of the respective processing PC.
– Port	Sets the port of the respective processing PC.
– Add	Adds the defined custom PC to the list of available PCs.
Refresh	Refreshes the list of available PCs.
Close	Closes this dialog.

12.9 EM Processing Toolbox

This module offers functionality for the processing of FIB-SEM stacks. This chapter describes how the different functions of the EM Processing Toolbox can be used to process a FIB-SEM-stack acquired with SmartFIB in the ZEN software. Note that parts of this special workflow also require functionalities of the **ZEN Connect** module. To make yourself familiar with this module, see also the documentation for *ZEN Connect [**▸ 562]*.

12.9.1 Licensing of EM Processing

For using the **EM Processing** functionalities, you need the license for the **EM Processing Toolbox** module.

Overview

Product Name	EM Processing Toolbox
ZEN lite	**n/a**
ZEN pro	**o**
ZEN desk	**o**
ZEN system	**o**
ZEN slidescan	**n/a**
ZEN celldiscoverer	**n/a**
ZEN SEM	**o**
ZEN image processing	**o**

x=included; **o**=optional; **n/a**=not available

12.9.2 Workflow Overview

This chapter gives an overview how you can process your FIB-SEM stacks and align them. Consider the following workflow:

1. **Sorting of image files:**
   With the function *Sort SmartFIB Tiffs [▸ 121]* you can sort your .tiff image files created by SmartFIB according to channel name, number of pixels, image size, and spacing of the images, corresponding to slice thickness. Note that this function only works if the tiff files have their default names (e.g. channel0_slice_0001.tiff or slice_0001.tiff)! Do not rename your files before you use this function!
2. **Image import and conversion:**
   With the special import functionality, you can import your FIB stack images and save them as a czi for further processing in ZEN. You can import the stack into ZEN (see *Importing SmartFIB Tiffs [▸ 360]*) or into **ZEN Connect** (see *Importing a SmartFIB stack into ZEN Connect [▸ 573]*).
3. **Subset image creation:**
   If you want to reduce the imported z-stack to a particular z-range and region before applying further processing steps, you can create a subset image of the imported FIB stack with the image processing function *Create Image Subset [▸ 112]*.
4. **Replacing individual slices in the z-stack:**
   If your stack contains image slices of bad quality which prevents further processing or segmentation, you can use the function *Slices Replacement [▸ 120]* to replace those slices with the respective predecessor or successor. For more information, see also *Replacing z-slices in a z-stack [▸ 358]*.
5. **Coarse alignment of the z-stack:**
   To minimize shifts in x and y in the z-stack and correct a potential beam shift, you can use the image processing function *Coarse Z-Stack Alignment [▸ 110]* to roughly align the z-stack. For more information, see also *Aligning z-planes manually [▸ 357]*.

6. **Image processing:**
   Use the *image processing functions [▸ 109]* to process your image and reduce artifacts. For general information about image processing, see also the chapter for the *Image Processing Workflow [▸ 90]*.
7. **Automatic alignment of the z-stack:**
   Make an automatic fine alignment of the planes in your z-stack with the processing function *Z-Stack Alignment with ROI [▸ 122]*. For more information, see also *Aligning z-planes automatically (based on a ROI) [▸ 358]*.
8. **Equalization:**
   Correct for variation of overall image intensity from image to image by equalizing the intensity value throughout the entire z-stack with the function *Z-Stack Equalization [▸ 123]*.
9. **Cropping of a specific volume:**
   Identify a particular region of interest and cut it out of your stack with the function *Cut Out Regions [▸ 113]*. For more information, see also *Cutting out a volume from a z-stack [▸ 359]*.
10. **Adding the processed z-stack to the ZEN Connect project:**
    Add your processed z-stack into the correlative workspace of the **ZEN Connect** module. For this you can use the **Add to Correlative Workspace** button in the toolbar, if you have an open project. In the correlative workspace you can then align several z-stacks and images (e.g. an overview image). For detailed information, see also *Adding an open image to the ZEN Connect project [▸ 568]* and *Aligning image data [▸ 576]*.
    In order to import the data into a specific session in the ZEN connect project, right-click on the respective session and select the czi file. The transformation that was applied to the session will then also be applied to the newly imported image.

12.9.3 Aligning z-planes manually

If you have an imported FIB-SEM stack which requires manual pre-alignment before using the automatic z-alignment, you can use the image processing function **Coarse Z-Stack Alignment** for a manual alignment of your stack. See the following instruction.

Prerequisite ✓ You have opened your (imported) z-stack that needs alignment in ZEN.

1. On the **Processing** tab, select the image processing function **Coarse Z-Stack Alignment**.
2. Click on the **Setup** button.
   → The *Coarse Z-Stack Alignment Setup [▸ 111]* opens.
   → The current z-plane (selected by the **Z-Position** slider in the **Dimensions** tab) is displayed in cyan and the following z-plane is displayed in red.
3. If you have an image with multiple channels, select the channel which should be displayed in the **Image View** with the **Channels** tool.
4. With the **Z-Position** control in the **Dimensions** tab, go to the z-plane where the image needs an alignment.
5. Select the **Speed** with which your alignment should be performed.
6. Shift the following z-planes with the arrow buttons in the top left of the setup until the x/ y shift in the z-stack seems to be eliminated. Alternatively, you can also use the arrow keys on your keyboard.
7. If necessary, adjust the speed step size during the alignment.
8. Repeat the steps 4 to 6 until all shifts in the z-stack are corrected.
   → Every shift is displayed in the **Shift List** on the left side of the setup.
9. Click on **Finish** to save the changes and close the setup.
   → The **Shift List** with all the shifts is displayed in the **Parameters** tool.
10. On the top of the **Processing** tab, click on **Apply**.

You have now aligned the planes in your z-stack manually to correct a shift in x and/ or y within the z-stack.

See also

Aligning z-planes automatically (based on a ROI) [358]

12.9.4 Aligning z-planes automatically (based on a ROI)

With the image processing function *Z-Stack Alignment with ROI [122]* you can perform an automatic alignment of the z-planes in a stack. This alignment can be based on a particular region of interest that you can draw into your image.

Prerequisite ✓ You have opened your z-stack in ZEN.

1. On the **Processing** tab, select the image processing function **Z-Stack Alignment with ROI**.
   → The method's options are displayed in the **Parameters** tool.
2. For **Quality**, select the quality for the image alignment.
3. For **Registration Method**, select whether the alignment to optimize the z-stack should perform a translation, a rotation, or both.
4. For **Interpolation**, select the interpolation method that should be used for the alignment.
5. Under **Region**, select **Rectangle Region** in the dropdown menu.
6. In the 2D view, draw a region of interest into your image to mark the structure(s)/ area of particular interest.
7. In the **Dimensions** tab, use the **Z-Position** slider to check if the region of interest needs to be adjusted. **Note:** The region of interest cannot be set for each plane individually. Make sure that the drawn region is big enough to contain the structure(s)/ area of interest in every z-plane.
8. Adjust the region of interest if necessary. You can adjust and move it directly in the 2D view or with the input fields in the **Parameters** tool.
9. If you want your output image to have the same size as your input image, activate the checkbox **Keep Size**. The image will be cropped in the process, in the case that pixels are shifted out of the area defined by the input image.
10. Under **Channel Component**, select one of the image channels whose alignment transformation matrix is then also applied to the other channel(s). If an alignment should be calculated for each channel individually, deactivate the checkbox **Single Component**. This step is only applicable for images with multiple channels.
11. At the top of the **Processing** tab, click on **Apply**.

The z-planes of your image are now aligned automatically. The progress of the alignment process is displayed in the progress bar on the bottom of the ZEN software.

12.9.5 Replacing z-slices in a z-stack

With the processing function *Slices Replacement [120]* you can replace slices of a z-stack with the previous or next slice in the stack.

1. On the **Processing** tab, select the method **Slices Replacement**.
2. In the Dimensions tab, use the **Z-Position** slider or input field to select the slice you want to replace.
3. Click on **Replace with Next** to replace the selected slice with the next one, or click on **Replace with previous** if you want to replace the slice with the previous one.
4. If you want to replace other slices as well, repeat the steps 2 and 3 for each slice.
   → Each slice is listed in the **Replacement Table** on the left.

5. Click on **Apply**.

You have now replaced the selected slice(s) with its previous and/or next one(s).

Replacing multiple slices

If you have several slices in your z-stack which you want to replace with the following or preceding slice, you can also take the following workflow:

1. On the **Processing** tab, select the method **Slices Replacement**.
2. Open the **Gallery** view of your z-stack.
3. In the **Gallery** view, press the **Ctrl** button and select all the slices you want to replace. As an example, you select the slices 34, 37, and 38.
4. Click on **Replace with Next** to replace the selected slices with the next ones, or click on **Replace with previous** if you want to replace the slices with the previous ones.
   → Each slice is listed in the **Replacement Table** on the left. As an example, clicking **Replace with previous** would replace slice 34 with 33, and 37 and 38 with the slice 36.
5. Click on **Apply**.

You have now replaced the selected slices with its previous and/or next ones.

12.9.6 Cutting out a volume from a z-stack

With the processing function *Cut Out Regions [▸ 113]* you can define a region in your z-stack and cut it out as a volume.

Prerequisite ✓ You have opened your z-stack in ZEN.

1. On the **Processing** tab, select the **Method** *Cut Out Regions [▸ 113]*.
2. Under **Parameters**, click on **Define Regions**.
   → The *Define Regions setup [▸ 114]* opens.
3. In the **Dimensions** tab, use the **Z-Position** slider to go to the first z-slice where you want to mark your region of interest.
4. In the *Region Edit section [▸ 114]* on the left, click on **Draw** and select the type of region you want to use (rectangular, circular, polygonal,...).
5. In the image, draw your so-called support region to mark the structure of interest.
6. Repeat the previous steps for the last slice where your structure of interest appears as well as for those slices in between where significant changes (in shape and/or position) of this structure take place.
7. Click on **Interpolate**.
   → Interpolated regions are created for all slices between the support regions. The interpolated regions are displayed with a slightly darker color.
8. Use the **Z-Position** slider to move through your stack and examine whether the interpolated regions satisfyingly cover the structure of interest in your stack.
9. If you find a slice where the interpolated region does not cover your structure of interest, draw a new support region on this slice and click on **Interpolate** again.
10. When the entire structure is marked satisfyingly by the interpolated regions, click on **Finish**.
    → The **Define Region** setup closes and your image with the newly created regions is displayed in the **Analysis** view.
11. On the **Processing** tab, click on **Apply**.

Your marked structure is now cut out of the z-stack and opened as a new image.

12.9.7 Importing SmartFIB Tiffs

In ZEN you can import SmartFIB stacks of Crossbeam microscopes. The orientation of these stacks differs from standard z-stack acquisition, as the acquired images are tilted by a certain angle compared to a z-stack acquired on a light microscope. The import function calculates this tilt from the metadata of the image. If the import finds no metadata concerning the tilt angle and the user does not enter a value for the sample angle, it uses a default angle of 54 degrees (default angle between FIB and SEM column at the Crossbeam) and the image is rendered with a 90 degree tilt when displayed in a ZEN connect project. Alternatively, you can enter the angle of your sample during import, e.g. as set during acquisition of the stack with SmartFIB, and the import then calculates the tilt angle based on this sample angle.
During import, the XY offset metadata of the individual slices is ignored by default and only the offset of the first tiff file is considered. This default avoids the creation of a slanted z-stack, however in certain cases, such as on-grid-thinning configuration, the XY offset of the individual slices needs to be taken into account.

1. On the **Processing** tab, select the image processing function **Import SmartFIB TIFFs**.
   → The function settings are displayed in the **Parameters** tool.
2. Click on **Select Files**.
   → A file browser opens.
3. Select the images you want to import as FIB stack.
   Note: Select only images with consistent metadata with respect to number of pixels, image size, and spacing of the images (i.e. use the Sort SmartFIB tiffs function before importing the data).
   Note: To make sure the stack is composed/ ordered correctly, watch out how the images are sorted in the explorer and in which order you choose them.
4. Enter a **File name** for the FIB stack.
5. If you import images without scaling information, deactivate the **Auto** checkbox for **XY-Scaling** and manually enter the information.
   Note: ZEN currently cannot determine automatically if scaling information is present.
6. You can set the slice distance manually if you deactivate the **Auto** checkbox for **Z-Spacing**. This step is optional and should only be done if you have reason to believe the information calculated with the metadata is incorrect. Leave the **Auto** checkbox activated and the slice distance is automatically calculated with information saved in the metadata of the images.
   Note: When you set the slice distance manually, the information in the metadata is ignored.
7. If you know the angle of your sample, deactivate the **Auto** checkbox for **Sample Angle** and enter it. Otherwise the tilt for the image is calculated using the metadata, or the sample angle is set to the default of 54 degrees (default angle between FIB and SEM column at the Crossbeam) and the image is rendered with a 90 degree tilt (if no information is available in the metadata).
8. If you want to consider the xy offset metadata of the individual slices for the import, activate the checkbox **Read XY Offsets**. Note that this can lead to a slanted z-stack depending on the sample and the metadata, assuming tilt correction was used during acquisition with SmartFIB (e.g. if the metadata contain incorrect offset information).
9. Click on **Apply**.

The FIB stack is now imported into ZEN and a czi-file is created.
Note: When importing larger image files, it may take a while until the entire stack is visible in the viewer.

12.10 Guided Acquisition

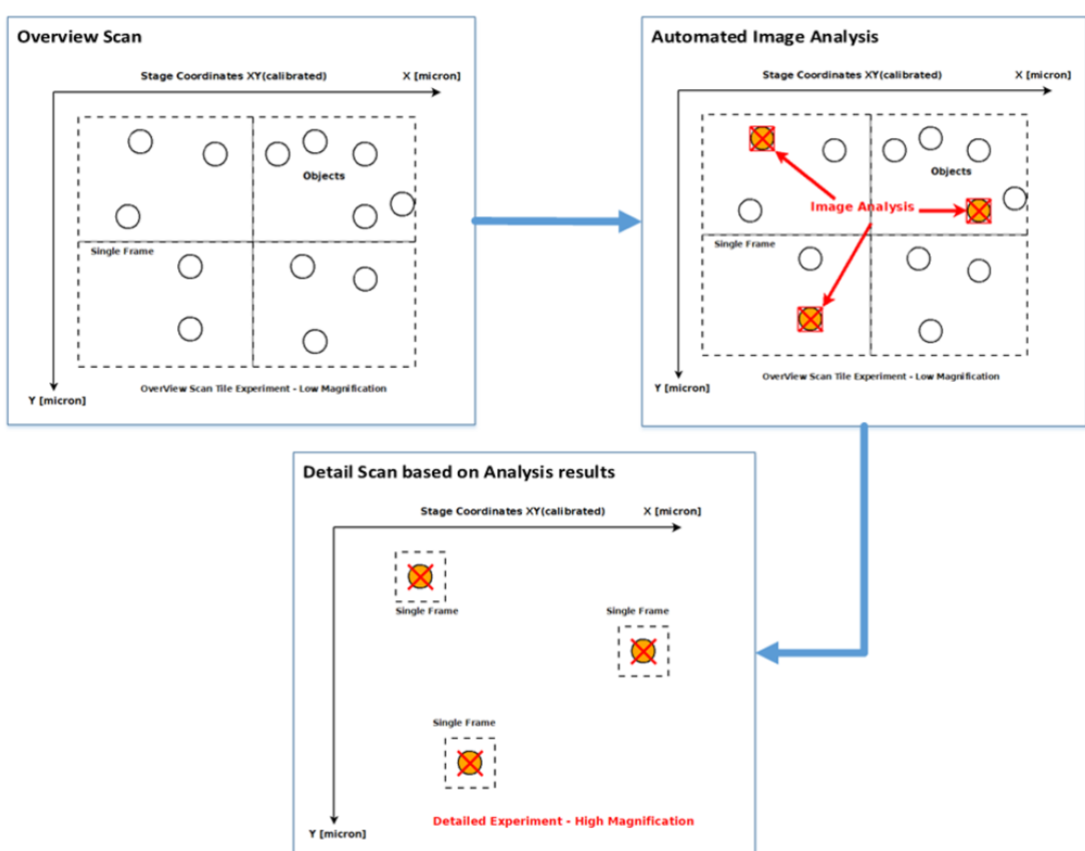
With the **Guided Acquisition** module for **ZEN (blue edition)** you can create an automated workflow to acquire images (overview), detect relevant objects (image analysis) and re-image these positions, using another experiment, e.g. with higher magnification, Z-stack etc.

Guided Acquisition Workflow:

1. Scan or inspect a large area (or over a long period of time).
2. Perform an analysis to detect interesting objects
3. Acquire detailed images for every detected object

A possible application is to detect rare events, e.g. to find transfected cells. For example, the sample contains many cells that are stained with a blue dye, but only a few are additionally expressing GFP. Guided Acquisition allows you to find these cells and run another, e.g. high-magnification, experiment on these positions.

After performing a (low-magnification) overview scan, the image analysis detects all cells and determines which of them are expressing GFP, i.e. show a certain intensity in the GFP channel. Then the microscope re-visits all GFP-expressing cells and performs a second acquisition there, e.g. with higher magnification, a Z-stack etc.



The analysis results are saved automatically to a folder.

12.10.1 Licensing of Guided Acquisition

For using the **Guided Acquisition**, you need the license for the **Guided Acquisition** module and the **Image Analysis** license.

Overview

Product Name	Image Analysis
ZEN lite	**o**
ZEN pro	**o**
ZEN desk	**x**
ZEN system	**x**
ZEN slidescan	**o**
ZEN celldiscoverer	**x**
ZEN SEM	**n/a**

x=included; **o**=optional; **n/a**=not available

12.10.2 Preliminary work to Guided Acquisition

For a successful **Guided Acquisition** experiment, you need to prepare an overview and a detailed experiment as well as an image analysis setting. If you want to process your overview image before it is analyzed, you also need a suitable setting for each processing step or function you want to execute.

Overview scan

You have defined the experiment for the overview scan. Typically, the overview scan is using a lower magnification in combination with a tile experiment.

Processing setting(s)

You have defined a suitable setting for each processing step or function you want to execute. For more information, see *General Settings [▸ 90]*.
Note that if you want to use Shading Correction with a reference image, you have to define your setting in Batch mode!

Image analysis setting

You have defined a suitable image analysis setting. For more information, see *Creating a new image analysis setting [▸ 369]*.

Detailed scan

You can perform for example the following experiments:

- simple Z-stacks with a high-NA objective lens
- Multi-Channel Z-stacks using an optical sectioning method like SD, Apotome or Airyscan
- Tile experiments

Parcentricity

If you use different detectors for the overview and the detailed scan, it might be necessary to correct for the shift between both detectors (to ensure parcentricity). For this you have to take an image at the exact same position with both cameras and then determine the offset between the two (to ensure parcentricity). The reference to calculate this offset is the image taken with the camera for the overview experiment. You can then enter the values for the shift in X and Y in the guided acquisition setup.

Focus strategies

You have several options, to perform a focus strategy.

Detailed scan and overview scan can be defined with their own focus strategy using **Focus Surface** and/or **Software Autofocus**.

During the Guided Acquisition experiment, you can define additional focusing steps. These are independent from the focus strategy defined on the **Acquisition** tab. For more information, see *Focus Strategy Tool [▸ 711]*.

For more information on Guided Acquisition, see *Performing a Guided Acquisition [▸ 363]*.

12.10.3 Performing a Guided Acquisition

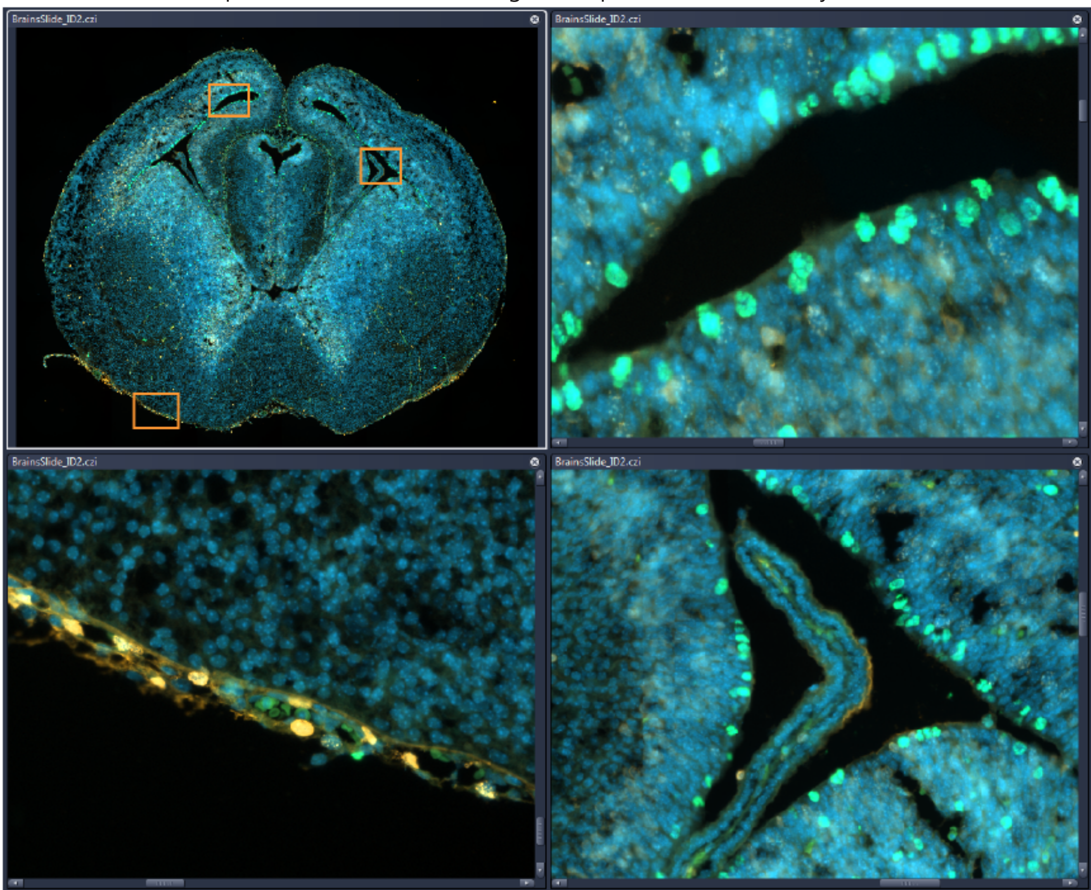
Prerequisite

- ✓ You have activated the **Guided Acquisition** module under **Tools > Modules Manager > Guided Acquisition**.
- ✓ You have calibrated the **XY stage**.
- ✓ You have defined a suitable experiment for overview scans and for detailed scans.
- ✓ You have defined a suitable setting for each processing step or function you want to execute.
- ✓ You have defined a suitable image analysis setting using the **Image Analysis Wizard** or an OAD macro that detects the objects of interest.
- ✓ For more information, see *Preliminary work to Guided Acquisition [▸ 362]*.

1. On the **Applications** tab, open the **Guided Acquisition** tool.
2. Create a setting to save your experiment setup. For more information, see *Using Guided Acquisition settings [▸ 365]*.
3. In the **Overview Scan** section, select the experiment you want to use for creating the overview image and the objective and (if available) after-magnification lens to be used. Note that as default objective and after-magnification are not stored as part of an experiment. For more information, see *Set up a new experiment [▸ 44]*.
4. If your system is equipped with Definite Focus, the **Find Surface** and **SW Autofocus** checkboxes are visible. Enable the checkbox to perform an additional focussing step before the Overview Experiment.
5. If you want to process your overview image before it is analyzed, click on **Add processing method**.
6. Select a **Method** for processing and a corresponding **Setting**.
7. In the **Image Analysis** section, select a suitable setting to analyze the overview scan and detect the objects of interest.
8. In the **Detailed Experiment** section, select the experiment for the detailed scan, the objective and - if available - the after-magnification lens to be used. Note that as default objective and after-magnification are not stored as part of an experiment. For more information, see *Set up a new experiment [▸ 44]*.
9. If you use different detectors for the detailed and overview experiments, enter a **X offset** and **Y offset** to correct the parcentricity.

10. If your system is equipped with Definite Focus, the checkbox **Find Surface** is available. Enable this checkbox to use the Definite Focus to find z-position of the glass surface once before the detailed scans. Check **SW Autofocus** to perform a SW autofocus once before the detailed scans. Optionally, check **Recall Focus** to store the difference between the glass surface and the sample and use this value for each of the detailed experiments.
11. Define the folder where you want to store the experiment data.
12. Click **Start**.

An overview scan is performed and a **.czi** image is acquired and saved to your folder.



In the example above, the image in the top left shows the overview scan and three identified objects marked with an orange box. For these positions, a detailed scan is performed with a higher magnification.

One Overview Scan Regions.csv and one OverviewScan Region.csv are displayed. The OverviewScan Region.csv table shows the found objects with **ID**, **Bound Center X Stage [µm]** and with **Y Stage [µm]**, **Bound Width**, and **Bound Height [µm]**, as well as **Image Scene Container Name** and **Image Index Scene**.

For each detected object a detailed scan is performed. For each object, a ***.czi** image is acquired and stored in your folder.

12.10.4 Using Guided Acquisition settings

Guided Acquisition offers you the possibility to save your whole experiment setup in a settings file. This file is saved in the folder for your Guided Acquisition experiment together with all the other settings (e.g. the overview and detailed experiment, the image analysis setting, and the image processing settings, if a processing step was selected).

Creating a Guided Acquisition setting

1. On the **Applications** tab, open the **Guided Acquisition** tool.
2. Click on the **Options** button and select **New**.
3. Name the setting and press *Enter* or click on .

You have created a setting for Guided Acquisition.

Saving a Guided Acquisition setting

When you have set up your Guided Acquisition experiment and created a setting, you can save the set up as a setting.

1. Click on the **Options** button and select **Save**.

Your experiment set up is now saved.

Importing and exporting a Guided Acquisition setting

1. On the **Applications** tab, open the **Guided Acquisition** tool.
2. Click on the **Options** button and select **Import** or **Export**.
   → A file browser opens.
3. Select the file you want to import or the folder where you want to export the setting to.
4. Click on **Open** / **Save**.

You have now imported/ exported a setting.

Deleting a Guided Acquisition setting

1. On the **Applications** tab, open the **Guided Acquisition** tool.
2. Select the setting that you want to delete in the drop down list.
3. Click on the **Options** button and select **Delete**.
4. Confirm that you want to delete the file.

The selected setting is deleted.

See also

- Performing a Guided Acquisition [363]
- Guided Acquisition Tool [366]

12.10.5 Functions & Reference

12.10.5.1 Guided Acquisition Tool

Parameter	Description
Options	
- New	Creates a new Guided Acquisition setting. Enter a name for the setting.
- Rename	Renames the setting.
- Save	Saves a modified setting under the current name. An asterisk indicates the modified state.
- Save As	Saves the current setting under a new name. Enter a name for the setting.
- Import	Imports an existing setting.
- Export	Exports the current setting.
- Delete	Deletes the current setting.
Overview Scan	
- Experiment	Selects the experiment setup to acquire the overview scan .
- Objective	Selects the appropriate objective for the overview scan (typically with a low magnification). If the objective has already been defined in the experiment, it is read only/ greyed out here.
- Optovar	Only visible if an optovar is available. Selects the optovar for after magnification of the overview scan. If the optovar has already been defined in the experiment, it is read only/ greyed out here.
- Find Surface	Only visible if Definite Focus is licensed. Performs a Find Surface before the overview scan. This step is independent from and additional to any focussing strategy defined in the overview experiment.
- SW Autofocus	Performs a SW Autofocus before the overview scan and sets the found z-position as z-position for the overview scan. This step is independent from and additional to any focusing strategy defined in the overview experiment.
- Initial Range	Only available if **SW Autofocus** is activated. Sets the range for the software autofocus.
Processing	Section for processing steps to process the overview image before it is analyzed.
- Add processing method	Adds a step for a new processing method.

Parameter	Description
- Method	Selects a processing method from a dropdown list. Available processing methods: ▪ Airyscan Processing ▪ ApoTome Raw Convert ▪ Deconvolution (defaults) ▪ Shading Correction ▪ Extended Depth of Focus
- Setting	Selects the setting for the processing method from the dropdown list. **Note:** If you want to use **Shading Correction** with a reference image, you have to define your setting in Batch mode!
- Remove	Removes this entire processing step.
Image Analysis	
- Setting	Selects the Image Analysis Setting used to analyze the overview scan.
Detailed Experiment	
- Experiment	Selects the experiment setup to acquire a detailed scan at the position of each object detected by the image analysis.
- Objective	Selects the appropriate objective for the detailed scan (typically with high magnification). If the objective has already been defined in the experiment, it is read only/ greyed out here.
- Optovar	Only visible if an optovar is available. Selects the optovar for after magnification of the detailed scan. If the optovar has already been defined in the experiment, it is read only/ greyed out here.
- Detector Parcentricity Correction	Only visible if the detectors used in the overview and detailed experiment are different. Sets a correction for parcentricity for the two detectors. In the two text fields you can enter the **X offset** and **Y offset** of the two different detectors in µm.
- Find Surface	Only visible if Definite Focus is licensed. Performs a Find Surface before the first detailed scan. This step is independent from and additional to any focussing strategy defined in the detailed experiment.

Parameter	Description
- SW Autofocus	Performs a SW Autofocus before the first detailed scan and sets the found z-position as z-position for the detailed scan. This step is independent from and additional to any focusing strategy defined in the detailed experiment. If the detailed experiment is not a tiles experiment the focus strategy is automatically set to the "standard" behavior, i.e. the selected autofocus strategy will be performed on each tile of each detailed experiment or each timepoint for a time-series, respectively. If you want to use a different strategy, e.g. perform the selected autofocus strategy on each n-th tile of each detailed experiment, you have to activate the **Tiles** checkbox for the detailed experiment. Then you can select the desired frequency of autofocus in the **Focus Strategy** tool under **Stabilization Event Repetitions and Frequency** when you switch to **Expert** mode.
- Initial Range	Only available if **SW Autofocus** is activated. Sets the range for the software autofocus.
- Recall Focus	Only visible if Definite Focus is licensed and if both **Find Surface** and **SW Autofocus** are checked. Restores the saved focus position and applies it for each detailed experiment.
Output Folder	
- Output Folder	Determines the folder where the analysis results are saved. A subfolder will automatically be created for each run of a Guided Acquisition experiment. **Note:** When a ZEN Connect project is open, the output folder cannot be set here, but is defined by the ZEN Connect project. **Note:** When using Guided Acquisition with Direct Processing, make sure that the processing computer has access to this output folder!
Open output folder after execution	**Activated:** Opens the output folder after Guided Acquisition is finished.
Start	Starts **Guided Acquisition**. The **Start** button turns into a **Stop** button as long as Guided Acquisition is running. Click on **Stop** to stop the running Guided Acquisition workflow.

See also

Software Autofocus Tool [▸ 507]

12.11 Image Analysis

This module enables you to create automatic measurement routines very easily. The Image Analysis wizard guides you through the steps to create an automatic measurement program. It allows you to set up even complex measurement tasks easily. The steps of the wizard include image segmentation, object separation and measurement of geometrical or intensity features. After you have completed the setup you can apply these settings to the data to be analyzed and obtain precise measurement results. You can display the results in table and list form and export them to csv-format.

For more information, see the following examples:

- *Measuring Fluorescence Intensity in a Multichannel Image [▸ 371]*
- *Counting Number of Fluorescence Signals per Nuclei [▸ 375]*
- *Measuring Mean Fluorescence Intensity on a Ring around the Primary Object [▸ 382]*
- *Counting the number of Objects in a Ring around the Nucleus [▸ 389]*

See also the following descriptions:

- *Image Analysis Tool [▸ 775]*
- *Image Analysis Wizard [▸ 777]*

12.11.1 Creating a new image analysis setting

When creating a new analysis setting for your images, you can select the following segmentation methods:

- **Segment region classes independently**: This method allows you to define several classes and subclasses. With this method, you can define the segmentation algorithm for each class independently.
- **ZOI (Zones of Influence)**: This method constructs a zone of influence (ZOIs) and a ring around each primary object. The primary objects are generated by segmenting the selected image channel with the selected class segmenter. The ring is defined by its width and distance from the primary object. The distance from the ZOI border from the ring can be specified. The ZOI area also incorporates the primary object and ring area.
- **Segment binary images**: This method allows you to segment binary images. With this method, you can define several classes and subclasses. The step **Automatic Segmentation** uses the defined binary image channels for each class to segment the image.
- **Whole image**: This segmenter uses the whole image as a region. You can use this to perform e.g. intensity measurements on the whole image without detecting objects. Therefore it is not possible to create more classes or subclasses.
- **Interactive Segmentation**: This method allows you to define multiple classes and subclasses. Unlike the other methods, it does not include an automatic segmentation step but uses only the interactive segmentation step. That means, for each class all regions must be drawn manually.

1. On the **Analysis** tab, in the **Image Analysis** tool, click on **Options** and select **New** from the dropdown menu.
2. In the **Settings** field, enter a name for your image analysis setting. Click on **Save**.
   → A new *.czias file is created and saved in the **...\ZEN\Documents\Image Analysis Settings** folder.
   → You have created a new image analysis setting. As default the method "Segment region classes independently" is used.
3. Click on **Edit** .

→ The **Segmentation Method Selection** dialog opens.

4. From the **Method** drop down menu, select a method and click on **OK**.

   → You have created a new image analysis setting using the method of your choice.

5. In the **Image Analysis** tool, click on **Setup Image Analysis**.

The **Image Analysis Wizard** opens with the **Classes** step and includes already a predefined set of classes, depending on the selected method. Follow the steps in the wizard to define your image analysis. Each method comes with a predefined set of steps which allows you to make all necessary settings for image analysis.

- **Classes:** Allows you to add classes and subclasses.
- **Frame:** Allows you to define a measurement frame. Only the area of the frame will be analyzed.
- **Region Filter:** Allows you to define simple or complex conditions to filter the detected objects according to their parameters.
- **Automatic Segmentation:** Allows you to set the parameters for the automatic segmentation.
- **Interactive Segmentation:** Allows you to modify the results of the automatic segmentation or draw/delete objects. Note: this step only generates relevant results in **Analyze Interactively** run.
- **Features:** Allows you to select measurement features from an extensive list and to define measurement features for classes and subclasses independently.
- **Results Preview:** Shows a preview of your measurement results for the current view port.

For more information, see the following examples:

- *Measuring Fluorescence Intensity in a Multichannel Image [▸ 371]*
- *Counting Number of Fluorescence Signals per Nuclei [▸ 375]*
- *Measuring Mean Fluorescence Intensity on a Ring around the Primary Object [▸ 382]*
- *Counting the number of Objects in a Ring around the Nucleus [▸ 389]*

12.11.2 Creating an image analysis setting from an analyzed image

It is possible to create / extract an image analysis setting from an image which has already been analyzed. This allows you to ensure that a new data set is analyzed exactly in the same way as a previously analyzed data set.

Prerequisite ✓ You have an already analyzed image.

1. Open your already analyzed image in ZEN.
2. On the **Analysis** tab, in the **Image Analysis** tool, click on Options .
3. In the drop down list, select **Create setting from analyzed image**.
4. Type in the name you want to give the analysis setting.
5. Press *Enter* or click on .

You have now created and saved an image analysis setting from an already analyzed image.

12.11.3 Measuring Fluorescence Intensity in a Multichannel Image

This topic will show you how to set-up a measurement program using the Image Analysis Wizard. After the setup is successfully completed, the program will be used to measure fluorescence intensity in a multichannel image.

In this example we are using a multichannel image with 2 channels (1st channel blue, (DAPI)), 2nd channel red (mRFP1)) of fluorescence-stained cells. First we detect the blue-stained cell nuclei in the first channel. Then we measure the fluorescence intensity for both channels for the detected nuclei.

See also

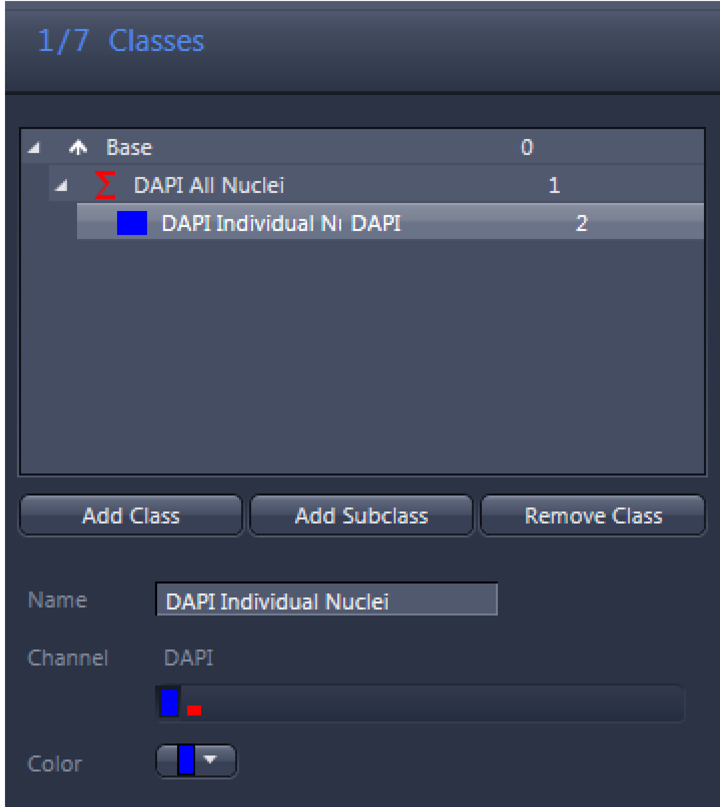
Creating a new image analysis setting [369]

12.11.3.1 Step 1: Classes

Prerequisite

- ✓ You have created a new image analysis setting with the **Segment region classes independently** method.
- ✓ You have opened the **Image Analysis Wizard**. For more information, see *Creating a new image analysis setting [369]*.

1. In the **Image Analysis Wizard 1/7 Classes**, click on **Class1** in the list and enter **DAPI Individual Nuclei** in the **Name** input field.
2. Click on **Classes1** in the list and enter **DAPI All Nuclei** in the **Name** input field.



3. Click on **Next**.

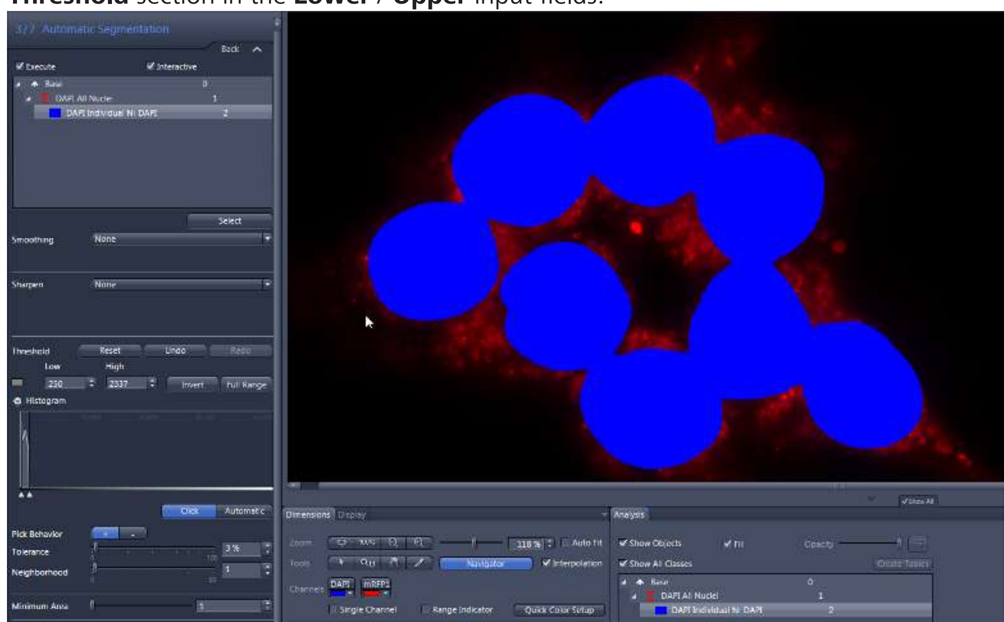
12.11.3.2 Step 2: (Measurement frame)

1. Deactivate the **Interactive** checkbox.
2. Click on **Next**.

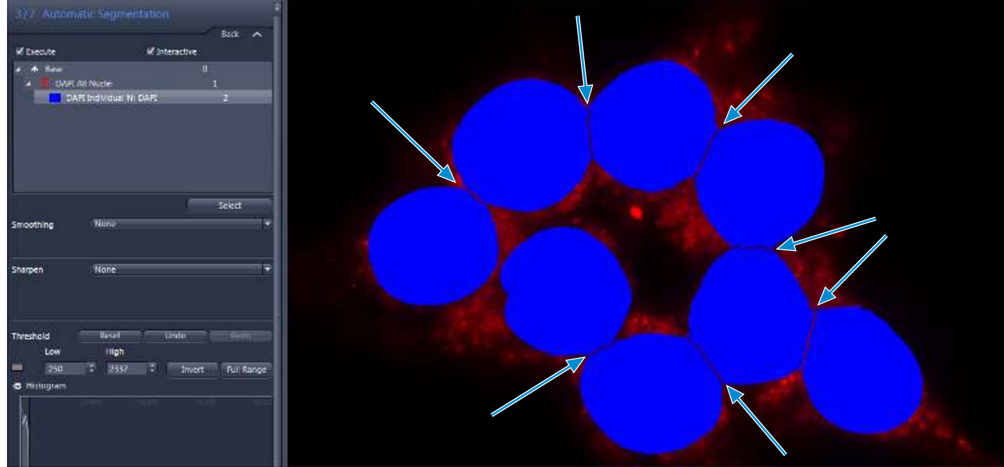
12.11.3.3 Step 3: Automatic segmentation

1. Click on **DAPI Individual Nuclei** entry in the list.
2. In the **Threshold** section set the **Tolerance** parameter to 1%.

3. Click in the image on the blue-stained cell nuclei.
   → The detected nuclei are overlaid in blue. The threshold values are displayed in the **Threshold** section in the **Lower** / **Upper** input fields.



4. Click on the areas of the blue cell nuclei that have not yet been detected until these have been completely overlaid.
5. Activate the **Fill Holes** checkbox.
   → This fills any holes in the detected cell nuclei.
6. Select the **Watersheds** entry from the dropdown list in the **Separate** section and set the number to 3.
   → Clear separation lines are now visible between the cell nuclei.



7. Click on **Next**.

12.11.3.4 Step 4: Region Filter

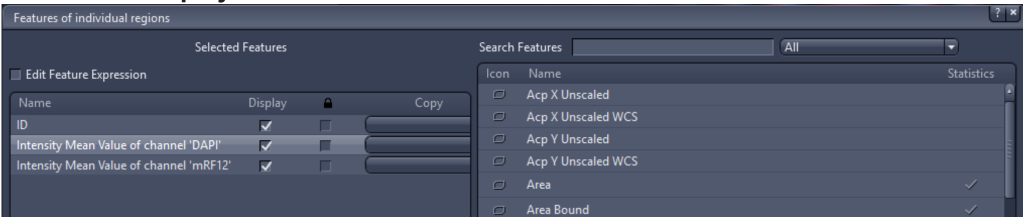
1. Deactivate the **Execute** checkbox.
2. Click on **Next**.

12.11.3.5 Step 5: Interactive segmentation

1. Deactivate the **Interactive** checkbox.
2. Click on **Next**.

12.11.3.6 Step 6: Features

1. Click on the **DAPI Individual Nuclei** entry in the list.
2. Click on the **Edit** button in the **Region Features** section.
   → The **Feature Selection** dialog is opened.
3. Double-click on **Intensity Mean Value of channel 'DAPI'** and **Intensity Mean Value of channel 'mRFP1'** features, one after another.
   → The features are displayed in the **Selected Features** list on the left.
4. Remove superfluous features (e.g. Area, Perimeter) from the list. Select the feature and click on the **Delete** button .
5. Activate the **Display** checkbox for the features.

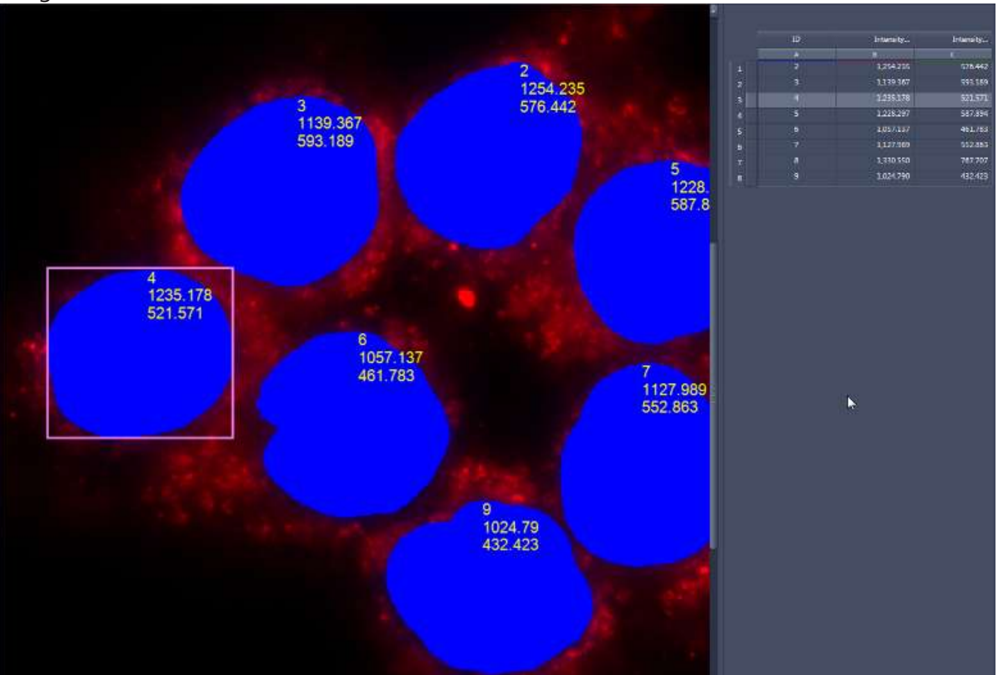


6. Click on **OK**.
   → The selected features are displayed in the **Region Features** section.
7. In the section **Annotation Options**, activate the checkbox **Color**.
8. Select **Yellow** from the drop-down list.
9. Click on **Next**.

12.11.3.7 Step 7: Results Preview

1. Click on **DAPI All Nuclei** in the list.
   → The number of measured cell nuclei is displayed in the data table to the right of the image.
2. Click on **DAPI Individual Nuclei** in the list.

→ The object ID and the values for the average fluorescence intensities per channel are displayed in the image at the cell nuclei in question and in the data list to the right of the image.

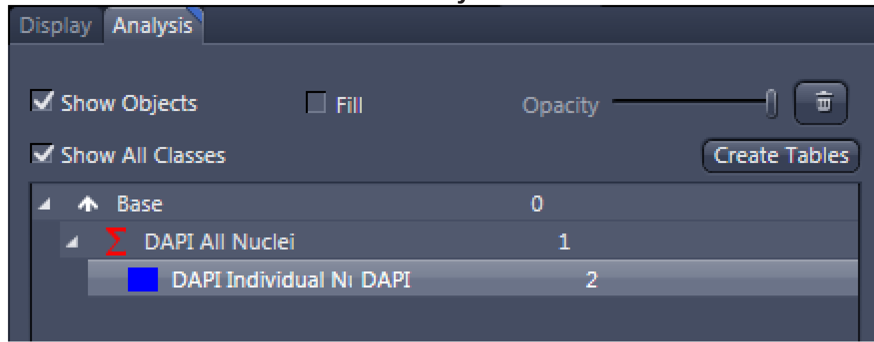


3. Click on the **Finish** button.
   → This saves the measurement program.

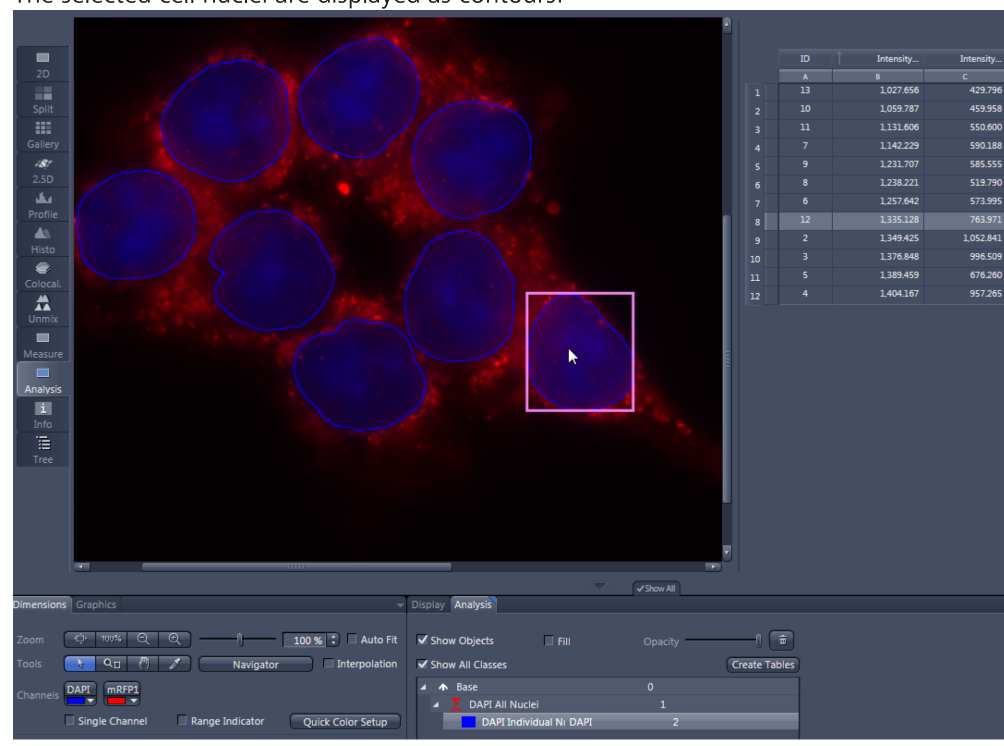
12.11.3.8 Executing the measurement program

Prerequisite
✓ You are in the **Image Analysis** tool.
✓ You have loaded the measurement program that you have generated.

1. Click on the **Analyze** button.
   → The measurement program is applied to the image.
   → The **Analysis View** now also appears in the **Center Screen Area**.
2. In the **Analysis View** you will see your image with the measured cell nuclei overlaid in blue and, to the right of this, the data list containing the individual measurements.
3. Deactivate the **Fill** checkbox in the **Analysis** control element.



→ The selected cell nuclei are displayed as contours.



4. Click on a row in the data list or alternatively on a cell nucleus in the image.
   → The row in the data list containing the measurement values is highlighted. The associated cell nucleus is surrounded by a red rectangle.

There is a direct link between the measured cell nuclei in the image and the measured values in the data table. You can either click on a measured cell nucleus in the image or on a row in the data table.l

12.11.4 Counting Number of Fluorescence Signals per Nuclei

This topic will show you how to set-up a measurement program using the Image Analysis Wizard. After this the program will be used to count the number of fluorescence spots in a multichannel image.
In this example we are using a multichannel image with 2 channels (1st channel blue (DAPI), 2nd channel green (GFP)) of fluorescence-stained cell nuclei. First we detect the blue-stained cell nuclei in the first channel and then the green stained signals in the second channel. Then we measure the number of green fluorescence signals per nucleus.

See also

Creating a new image analysis setting [369]

12.11.4.1 Step 1: Classes

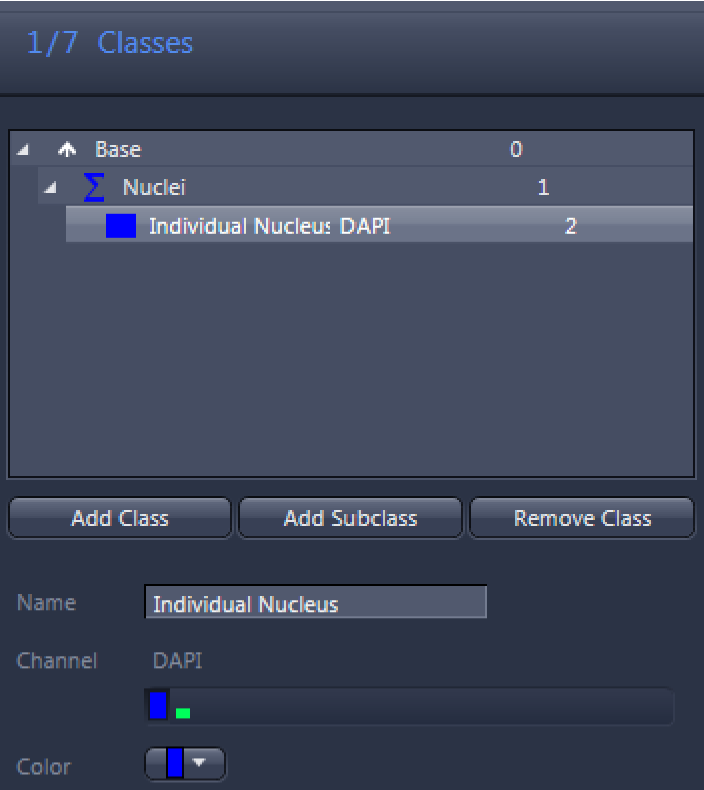
Prerequisite

✓ You have created a new image analysis setting with the **Segment region classes independently** method.

✓ You have opened the **Image Analysis Wizard**. For more information, see *Creating a new image analysis setting [369]*.

1. In the**1/7 Classes**, click on **Classes1** in the list and enter **Nuclei** in the **Name** input field.
2. Select a blue color from the dropdown list in the **Color section**.

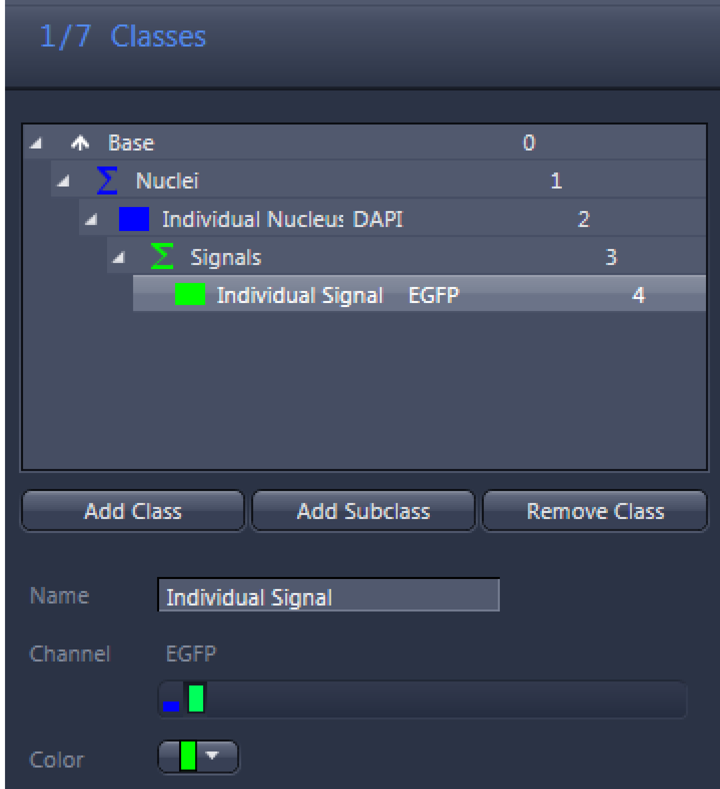
3. Click on the **Class1** entry in the list and enter **Individual Nucleus** in the **Name** input field.
4. Click on the blue channel icon in the **Channel** section .



→ You have now setup a class pair for the nuclei.

5. Click on the **Add Subclass** button .
6. Click on **Classes3** in the list and enter **Signals** in the **Name** input field.
7. Select a green color from the dropdown list in the **Color** section
8. Click on the **Class3** entry in the list and enter **Individual Signal** in the **Name** input field.

9. Click on the green channel icon in the **Channel** section.



→ You have now setup a subclass for the signals inside the Individual Nucleus class (parent class).

10. Click on **Next**.

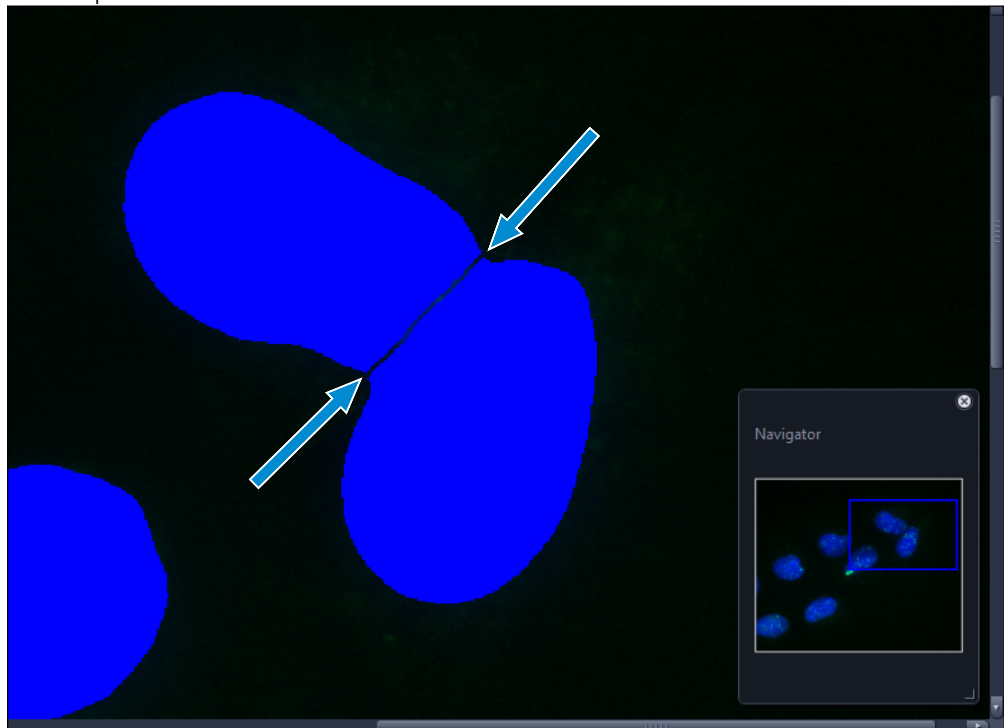
12.11.4.2 Step 2: Measurement frame

1. Deactivate the **Interactive** checkbox.
2. Click on **Next**.

12.11.4.3 Step 3: Automatic Segmentation

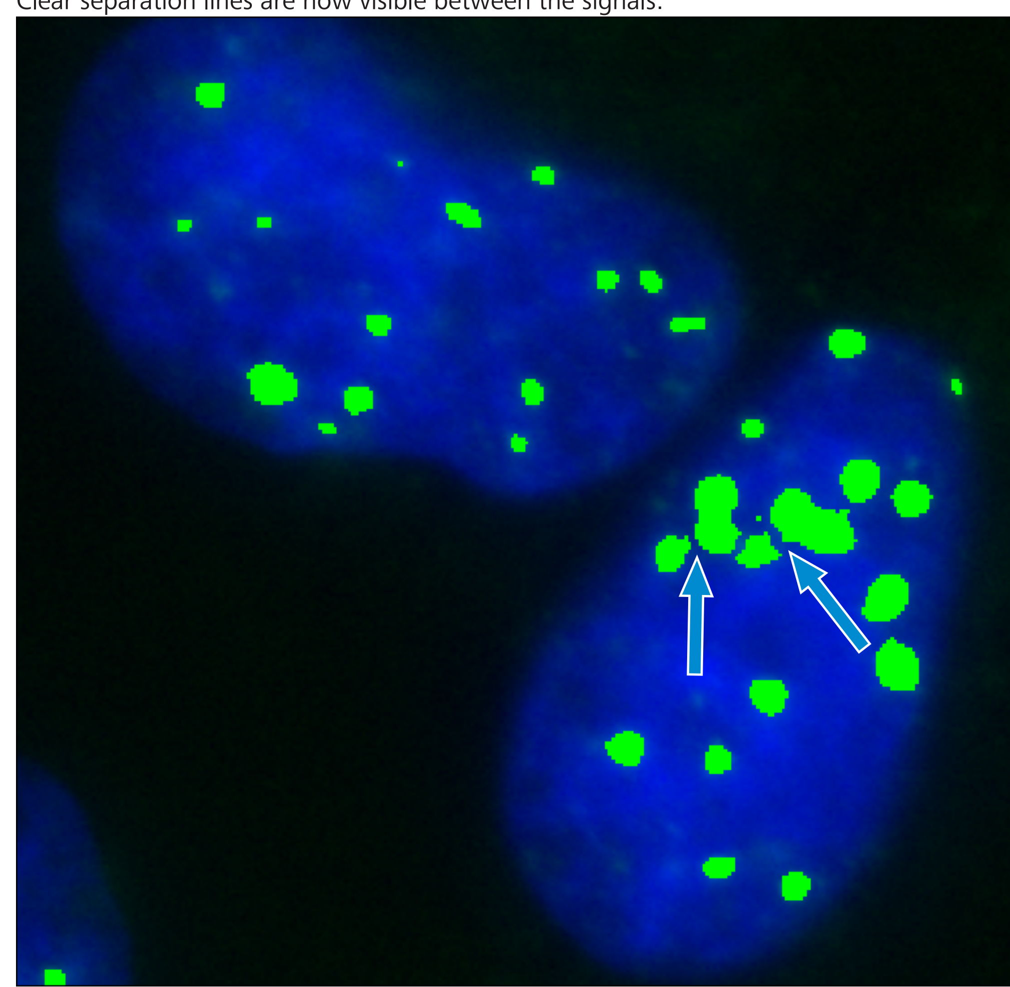
1. Click on the **Individual Nucleus** entry in the list.
   → The segmentation parameters (**Smooth**, **Sharpen**, **Minimum Area**, etc.) are displayed below the list.
2. In the **Smooth** section select **Gauss** from the dropdown list and set the parameter **Sigma** to **1.5**.
3. Click on the blue-stained cell nuclei in the image.
   → The detected nuclei are overlaid in blue. The threshold values are displayed in the **Threshold** section in the **Lower** / **Upper** input fields.
4. Click on the areas of the blue cell nuclei that have not yet been detected until these have been completely overlaid.
5. Select the **Watersheds** entry from the dropdown list in the **Separate** section and set the number to **17**.

→ Clear separation lines are now visible between the cell nuclei.



6. Click on the **Individual Signal** entry in the list.
   → The segmentation parameters (**Smooth**, **Image Sharpness**, **Minimum Area**, etc.) are displayed below the list.
7. In the **Smooth** section select **Gauss** from the dropdown list and set the parameter **Sigma** to **1.5**.
8. Click in the image on the green-stained signals.
   → The detected signals are overlaid in green. The threshold values are displayed in the **Threshold** section in the **Lower** / **Upper** input fields.
9. Click on the areas of the green signals that have not yet been detected until these have been completely overlaid.
10. Activate the **Fill Holes** checkbox.
11. This fills any holes in the detected signals.
12. In the **Separate** section select the **Watersheds** entry from the dropdown list and set the number to **17**.

→ Clear separation lines are now visible between the signals.



13. Click on **Next**.

12.11.4.4 Step 4: Region Filter

1. Deactivate the **Execute** checkbox.
2. Click on **Next**.

12.11.4.5 Step 5: Interactive Segmentation

1. Deactivate the **Interactive** checkbox.
2. Click on **Next**.

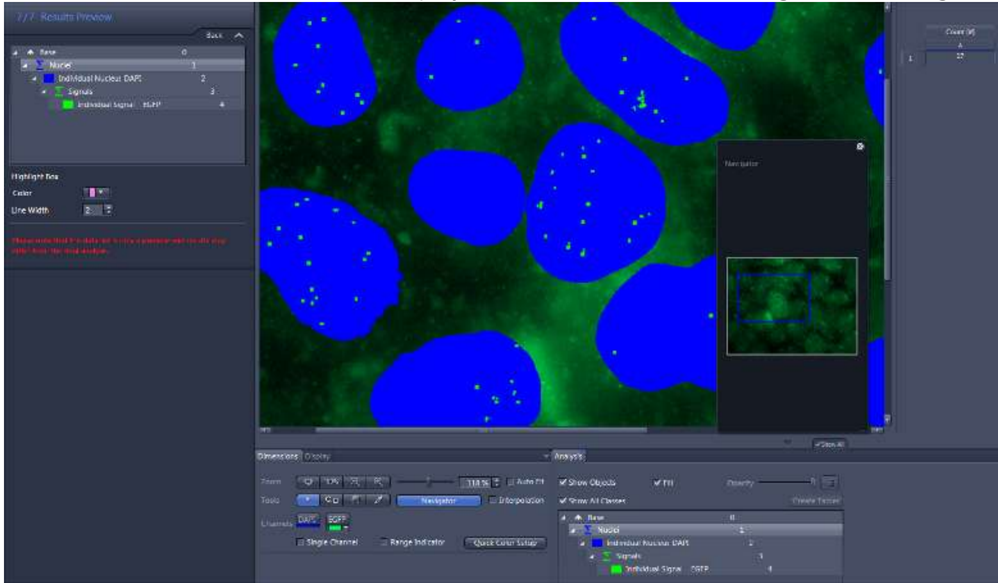
12.11.4.6 Step 6: Features

1. Click on the **Nuclei** entry in the list.
2. Click on the **Edit** button in the **Regions Features** section.
   → The **Feature Selection** dialog is opened.
3. Double-click in the right-hand list on the **ID**.
   → The features are displayed in the **Selected Features** list on the left.
4. Remove superfluous features from the list. Select the feature and click on the **Delete** button.
5. Click on the **Individual Nucleus** entry in the list.

6. Click on the **Edit** button in the **Features of individual regions** section.
   → The **Feature Selection** dialog opens.
7. Double-click in the right-hand list on the **ID of the parent**.
   → The features are displayed in the **Selected Features** list on the left.
8. Remove superfluous features (e.g. Area, Perimeter) from the list. Select the feature and click on the **Delete** button.
9. Click on the **Signals** entry in the list. This list contains the statistical features of the individual signals.
10. Click on the **Edit** button in the **Regions Features** section.
    → The **Feature Selection** dialog opens.
11. Double-click in the right-hand list on the **ID, Count**.
    → The features are displayed in the **Selected Features** list on the left.
12. For the newly added feature **Count**, open the **Copy** drop-down menu and select **Copy to the parent single region**.
    → The feature is copied to the results table of the parent, in this case the individual nucleus.
13. Remove superfluous features from the list. Select the feature and click on the **Delete** button.
14. Click on the **Individual Signal** entry in the list.
15. Click on the **Edit** button in the **Features of individual regions** section.
    → The **Feature Selection** dialog opens.
16. Remove superfluous features (e.g. Area, Perimeter) from the list. Select the feature and click on the **Delete** button.
17. Click on the **OK** button.
    → The selected features are displayed in the **Regions Features** section.
18. Click on **Next**.

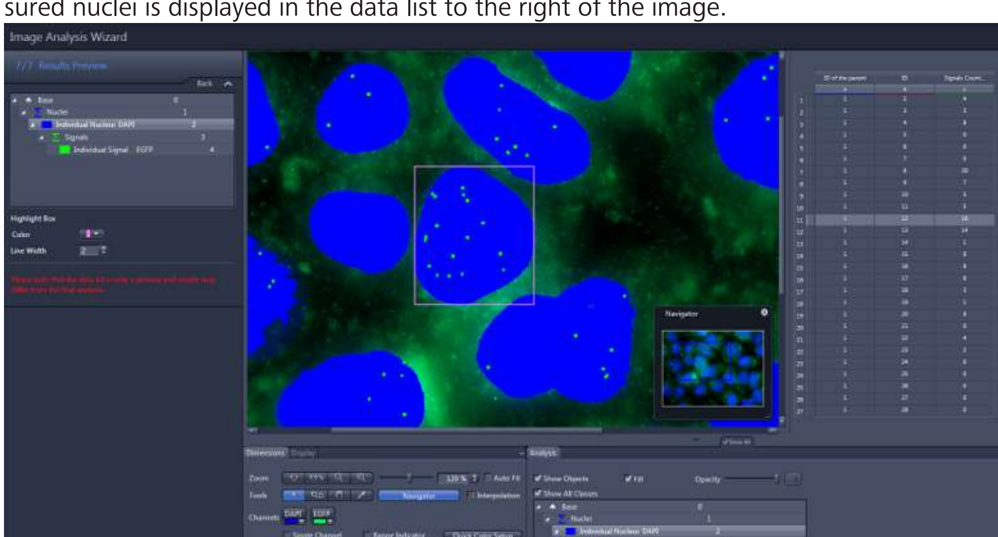
12.11.4.7 Step 7: Results Preview

1. Click on **Nuclei** in the list.
   → The number of measured nuclei is displayed in the data table to the right of the image.



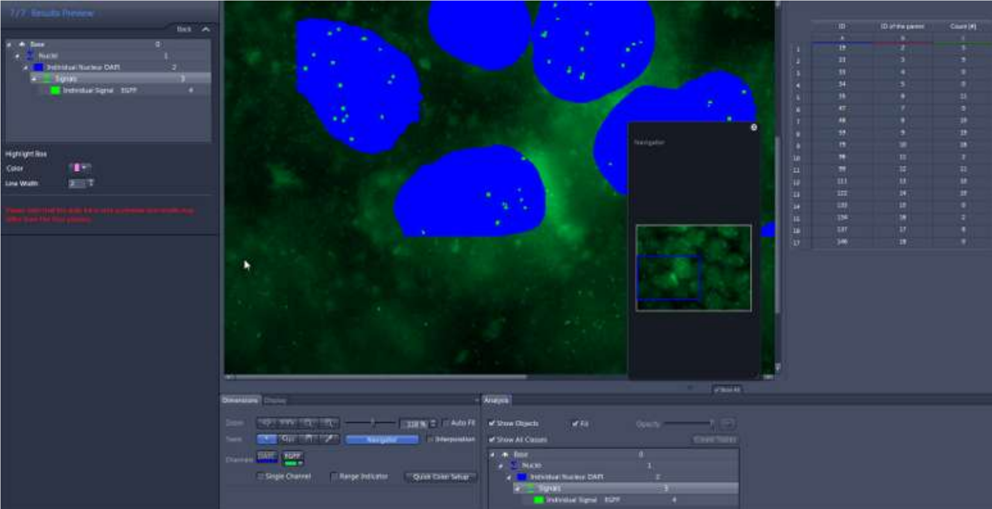
2. Click on **Individual Nucleus** in the list.

→ The ID of the parent, the ID and Signals Count (the number of green signals) of the measured nuclei is displayed in the data list to the right of the image.



3. Click on **Signals** in the list.

→ The **ID of the parent** (corresponds to the ID of the nucleus) and the number of measured signals are displayed in the data table to the right of the image.



4. Click on the **Finish** button.

→ This saves the measurement program.

12.11.4.8 Executing the measurement program

Prerequisite
- ✓ You are in the **Image Analysis** tool.
- ✓ You have loaded the measurement program that you have generated.

1. Click on the **Analyze** button.

→ The measurement program is applied to the image.

→ The **Analysis View** now also appears in the **Center Screen Area**.

→ In the **Analysis View** you see your image with the measured cell nuclei overlaid in blue and the signals overlaid in green. Right of this, you see the data list containing the number of signals per nucleus.

→ Only the number of signals of measured nuclei is displayed. Nuclei touching the frame are not taken into account.

12.11.5 Measuring Mean Fluorescence Intensity on a Ring around the Primary Object

The following example shows how to use the Zone of Influence (ZOI) method to measure intensities within a ring that is associated to the main object, e.g. the cell nucleus. An application example are transport assays where the intensities of a certain fluorescent marker in the cytoplasm are compared to the intensities within the nucleus.

In this example we use a multichannel image of fluorescence-stained cells. The cell nuclei are stained with AF568 and the mitochondria are stained with AF488. First, we detect the nuclei in the AF568-channel as primary object. A zone of influence is generated around each detected primary object. In this area, we can define a ring and specify its thickness and distance from the main object. You can use this ring to measure intensities or to detect further sub-objects on it. For more information, see *Counting the number of Objects in a Ring around the Nucleus [▸ 389]*.

See also

Creating a new image analysis setting [▸ 369]

12.11.5.1 Step 1: Classes

Prerequisite

✓ You have set up the image analysis setting with the method **ZOI (Zones of Influence)**. This has created the classes **ZOIs/ZOI**, **Primary Objects/Primary Object**, and **Rings/Ring** by default.

✓ You have opened the **Image Analysis Wizard**. For more information, see *Creating a new image analysis setting [▸ 369]*.

1. If you want to extend the predefined list of classes, in the **Image Analysis Wizard 1/6 Classes**, click **Add Subclass**.
   → You can find an example how to detect objects on the ring in *Counting the number of Objects in a Ring around the Nucleus [▸ 389]*.
2. If neccessary, click **Add Class** to extend the predefined list by another independent class of objects.
   Note that you cannot add further rings.
3. Select the image channel which you want to use for object detection. In this example, the primary objects (the nuclei) are in the AF568 channel. Therefore, click on the class **Primary Object** and select the channel containing the nuclei.

Parameter	Description
ZOIs	Class of all zone of influences
ZOI	Individual zone of influence
Primary Objects	Class of all primary objects
Primary Object	Individual primary object
Ring	Individual ring
Ring Segment	Part of a ring (a ring often consists of only one ring segment)
Add Class	Adds a new independent class (under base).
Add Subclass	Adds a new subclass to primary object, ring or another class.
Remove Class	Removes the selected class or subclass.
Name	Name of the classes. You can rename them, if necessary.
Channel	Select the channel to be used for object detection

Parameter	Description
Color	Changes the color how the classes will be displayed.

12.11.5.2 Step 2: Frame

Optionally, you can define the area to be analyzed of each image. In case there are shading effects or other reasons that make you want to include only a certain area of each image for analysis, you can define a frame (rectangle, circle or polygon). Only the area within this frame will be further analyzed.

With the **Mode** parameter, you can furthermore choose how the analysis treats objects that are cut by the border of the image or the frame:

- **Cut at frame**: Cuts the objects at the frame.
- **Inside only**: Discards all objects that are cut by the frame.

Note that the whole ZOI is taken into account for the decision if the object is inside the frame or not (not only the primary object). This means there are cases where the primary object might lie completely within the image / frame, but the Ring or ZOI is cut by the border. If you select **Inside only** these objects will be discarded.

Comparison of image analysis results for **Cut at frame** compared to **Inside only**:

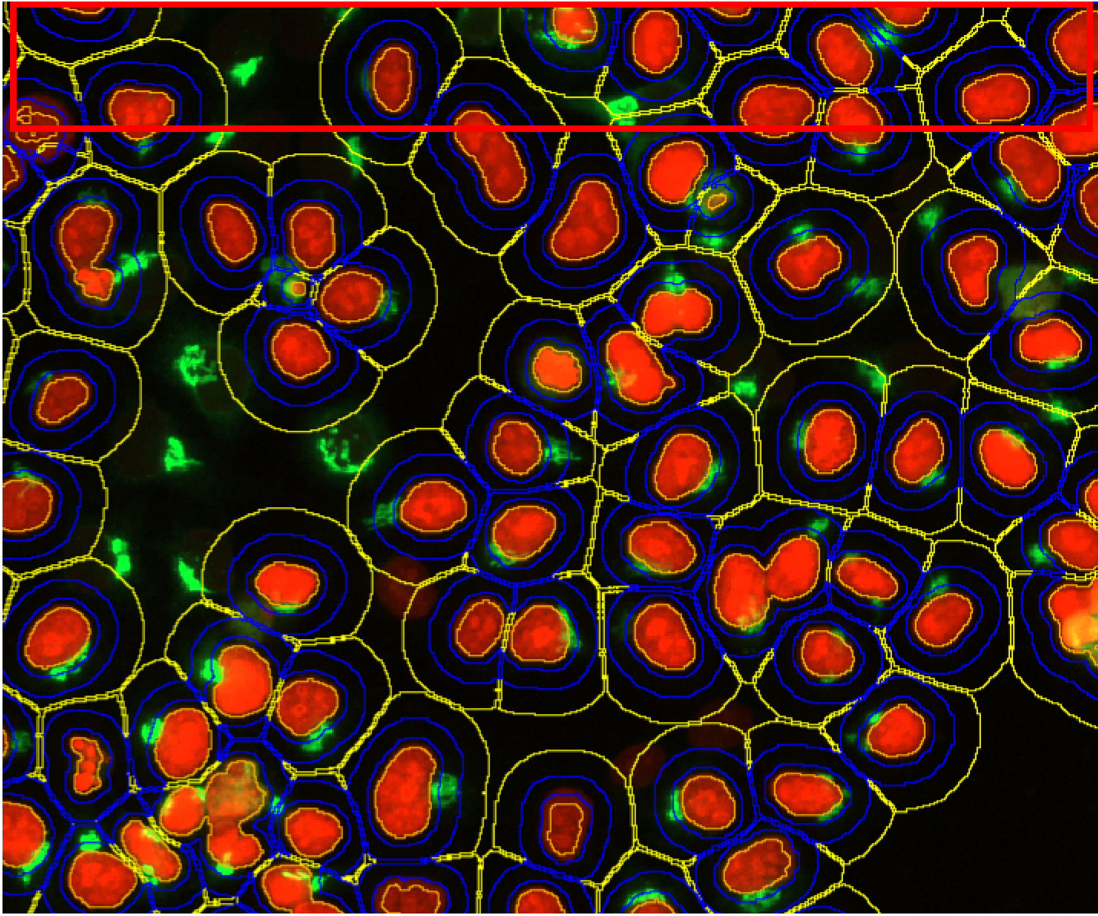


Fig. 16: Cut at frame

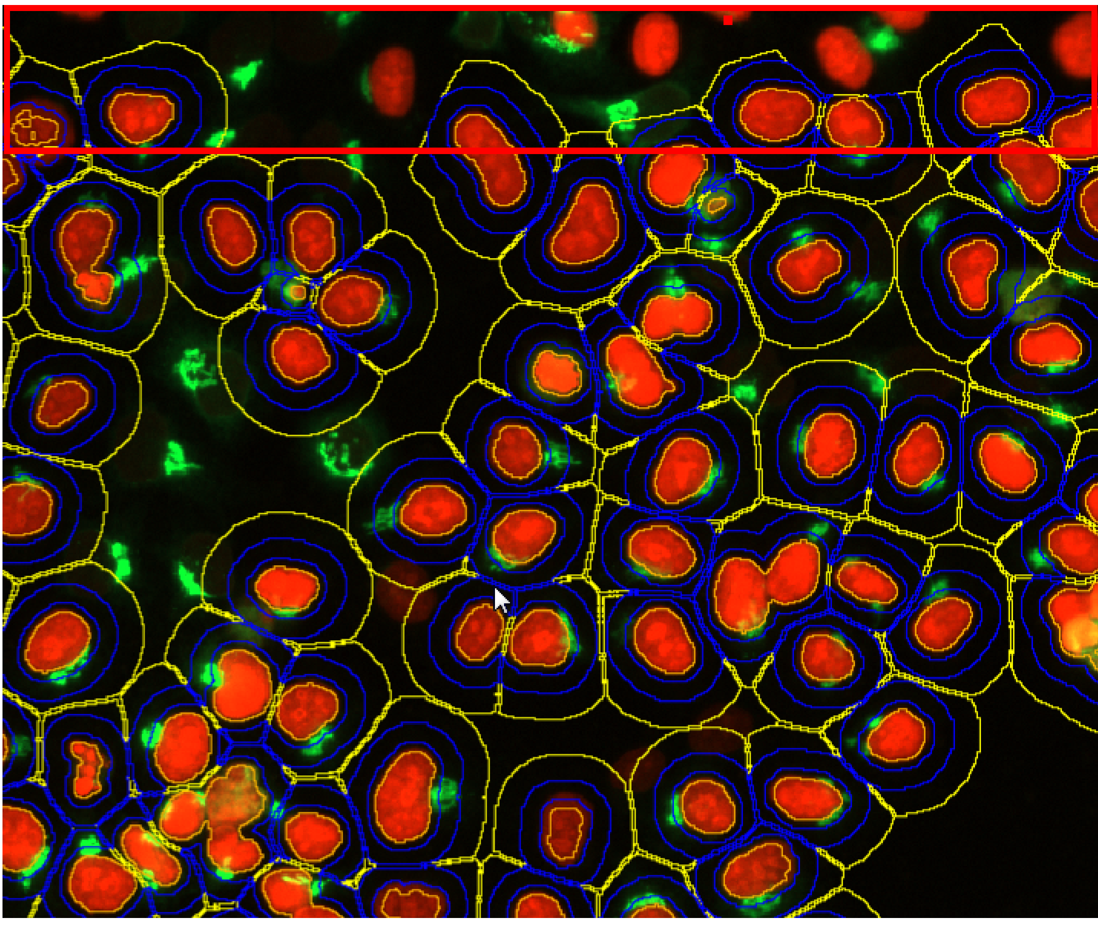


Fig. 17: Inside only

12.11.5.3 Step 3: Automatic Segmentation

1. Select **Primary Object** and set up suitable parameters to detect the objects, i.e. threshold, area, separation.
   → As soon as objects are detected, the ZOI and Ring are automatically created around each primary object with the preset parameters.
2. To modify **Ring Distance** and **Width**, select the **Ring Element** class.
   → Now, you can define the location and dimension of the ring flexibly. You can set it at the edge of the main object or inside the main object. You can also define an arbitrary distance.
3. Define the following parameters:
   → **Ring Distance**: Distance from surface of the primary object. Negative values means that the ring starts at the defined distance within the primary object. **Ring Width**: Defines the width of the ring.
   → The ZOI is automatically adapted to exceed the class with the larger diameter, i.e. either ring or primary object, by at least 3 pixel (default setting).

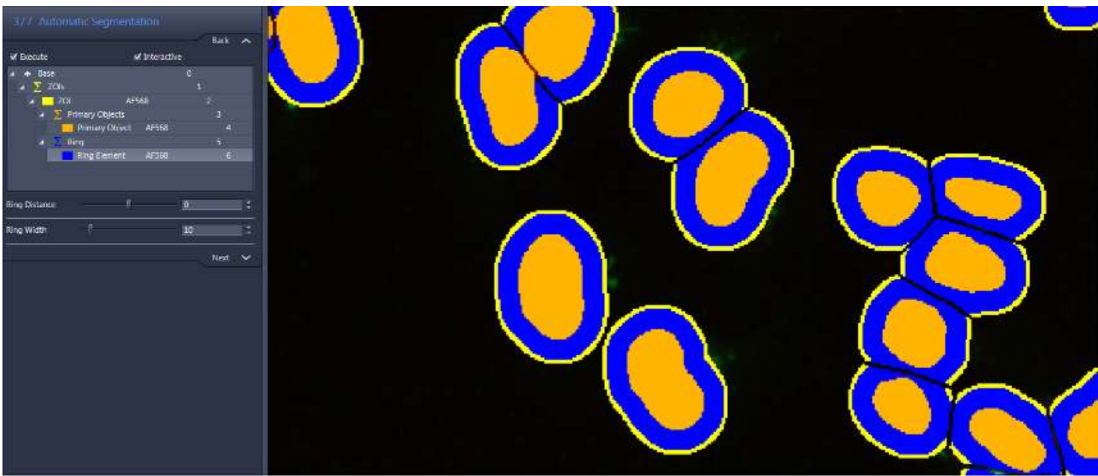


Fig. 18: Ring distance: 0 pixel; Ring width: 10 pixel

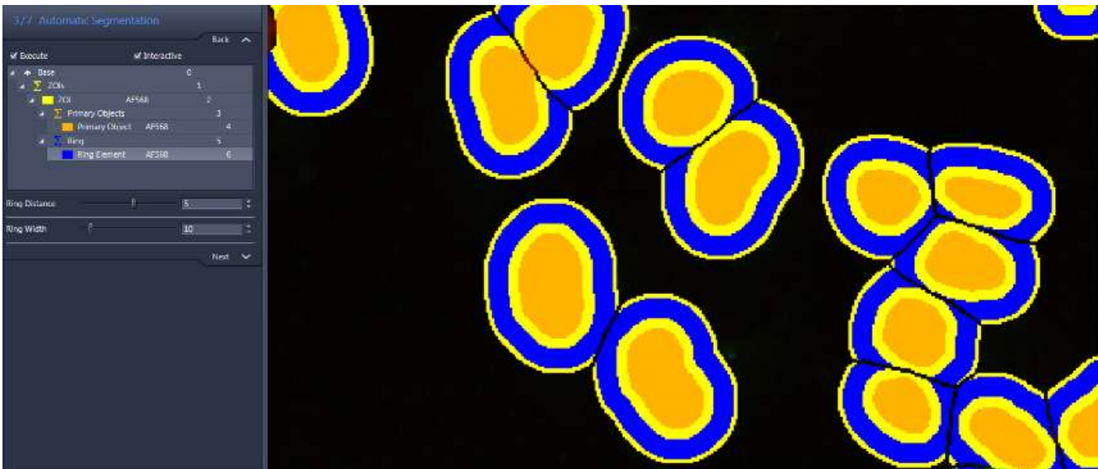


Fig. 19: Ring distance: 5 pixel; Ring width: 10 pixel

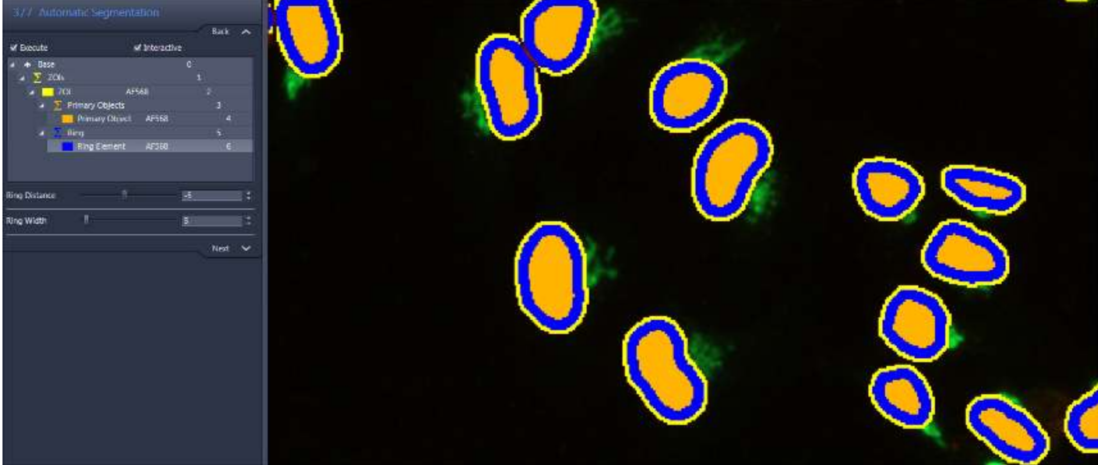


Fig. 20: Ring distance: -5 pixel; Ring width: 5 pixel

Modifying the ZOI Width

1. Select **ZOI-class**, and with the **ZOI Width** slider, set the distance. You can set the distance between the outer border of the ZOI and the outer border of either ring or primary object, respectively. The **ZOI Width** is at least 3 pixels larger than either the ring or the primary object, whichever is larger. The ZOI area incorporates also the area of the ring and the primary object, and thus can serve for example for measuring features over the complete cell.

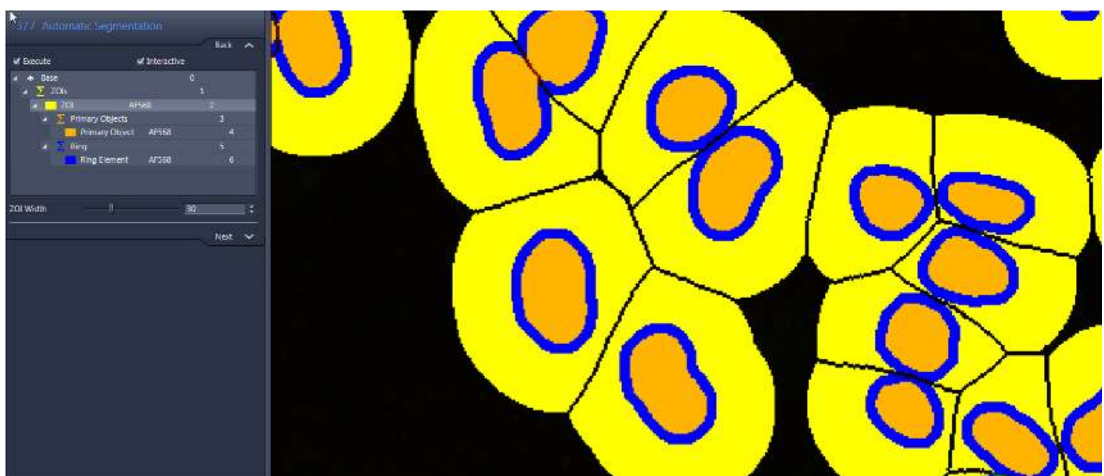


Fig. 21: ZOI Width set to 30 pixel

12.11.5.4 Step 4: Region Filter

You can define conditions for the primary objects (and additionally defined subclasses) to be measured, e.g. include only objects of a certain size, shape, intensity or other parameters. You can define suitable parameters for each of the defined objects.

1. Select the **Primary Object** and click **Edit**. From the list of features on the right, you can add features via double-click. Once you have added all desired features, click **OK**.
   → The selected conditions appear in the left tool area.
2. Set the minimum and maximum values by clicking on the objects with the desired features, or by entering the numbers directly.

The following figure is an example and shows the result if a certain condition of the circularity of each primary object needs to be fulfilled.

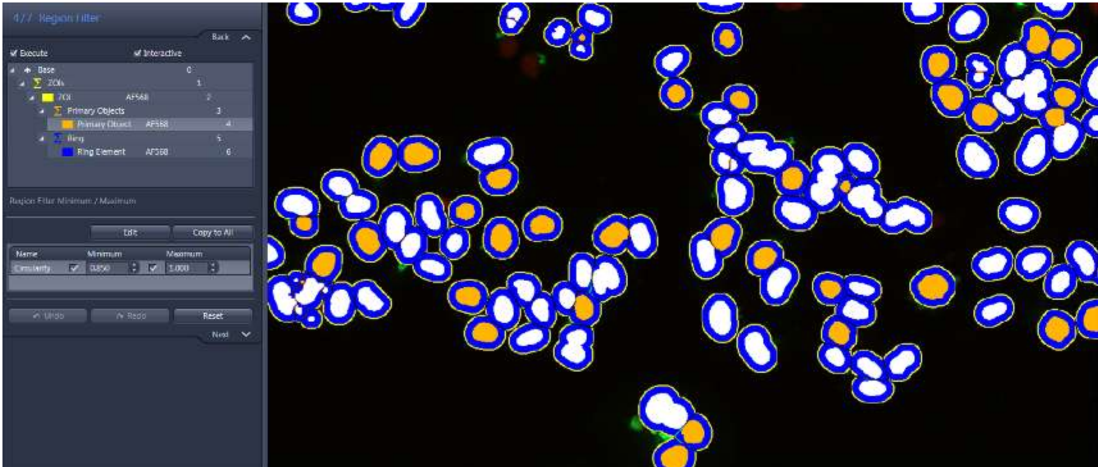
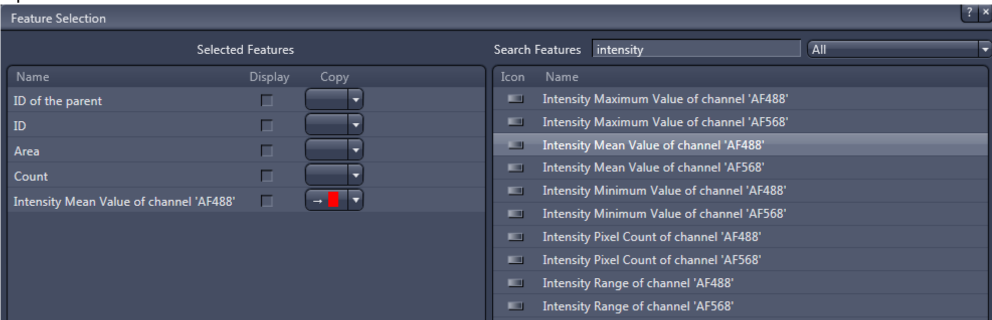


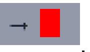
Fig. 22: Region Filter based on the circularity of the primary object

12.11.5.5 Step 5: Features

You can define individual measurement features for each class. You can copy the measurement features defined for one class to the other classes via **Copy to all**.

1. Select the class for which you want to define measurement features, and click **Edit**. From the list of features on the right you can add features to the selected features list on the left.
   → These features are automatically calculated for every object during image analysis. All classes have the ID of the parent and ID as default features. With these IDs you can later group the associated parameters from the result excel lists, if necessary.
   → The class **Ring** additionally has **Area** and **Count** as default parameters.
   → To attribute the mean intensity for channel AF488 (the mitochondria) measured on the **Ring** to the **Primary Object**, select **Ring** and click **Edit** The **Feature Selection** window opens.



2. From the feature list on the right select **Intensity Mean Value of channel 'AF488'** and add it to the selected features on the left. In the **Copy** column, from the drop-down menu in the **Intensity Mean Value of channel 'AF488'** row, click .
   → This measurement feature is copied to the corresponding **Primary Object**.

12.11.5.6 Step 6: Result Preview

In this step you see a preliminary result of the image analysis.

1. Click on the different objects in the **Analysis** tab to get the preliminary measurement result for all objects.
2. Click **Finish**, to save the analysis settings and close the wizard.
   → The wizard closes. The analysis settings are saved.

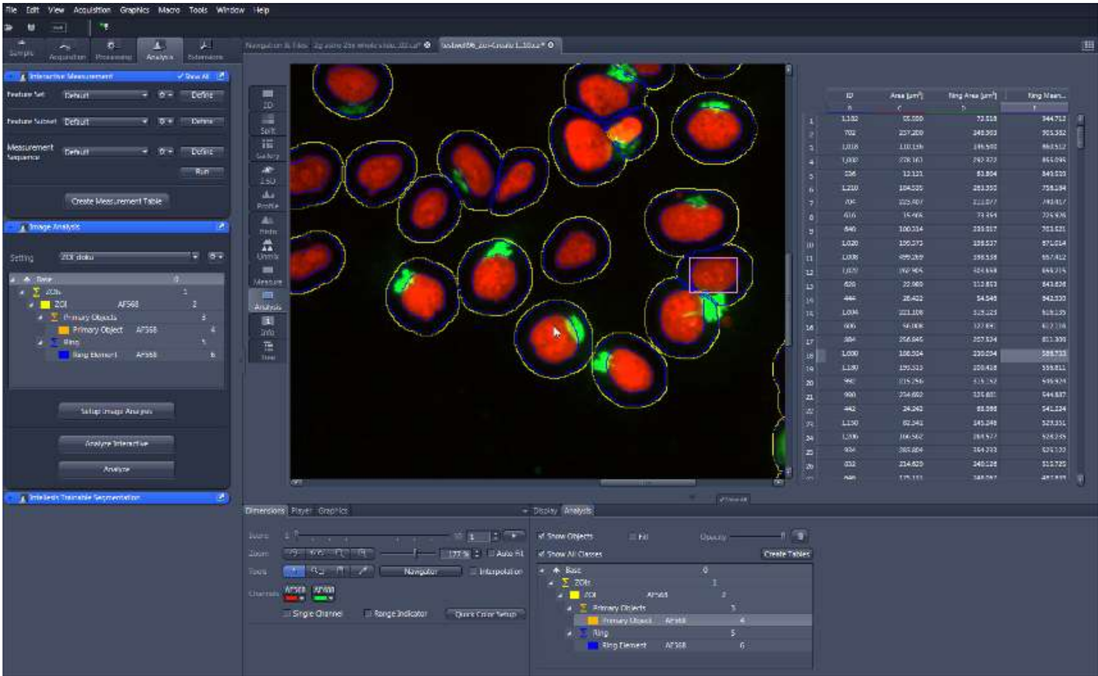
12.11.5.7 Executing the measurement program

You have the following options to run a predefined image analysis setting on your data set:

- **Analyze Interactively**: Analyze interactively with all steps that have been selected with the checkbox **Interactive** during setup of the image analysis.
- **Analyze**: Runs the image analysis setting without dialog.

When the analysis is finished, the main view switches to the **Analysis** tab and displays the segmented image along with the results of the analysis.

Select the different objects to display the corresponding measurement tables. The data in the tables and the regions in the image are interlinked. A click on the object in the image highlights the corresponding line in the data table and vice versa.



12.11.6 Counting the number of Objects in a Ring around the Nucleus

This example is similar to *Measuring Mean Fluorescence Intensity on a Ring around the primary Object [▸ 382]* and also uses the same data. This example shows how to count the number of objects on a ring that is associated with the main object, e.g. the cell nucleus. The images are taken from AF568 stained nuclei. The mitochondria are stained with AF488. The channel of the nuclei is used for image segmentation. The ZOI-segmentation method attributes a zone of influence (ZOI) and a ring to each detected nucleus. This area is used as a search range to detect subobjects, in this case the mitochondria.

See also

Creating a new image analysis setting [▸ 369]

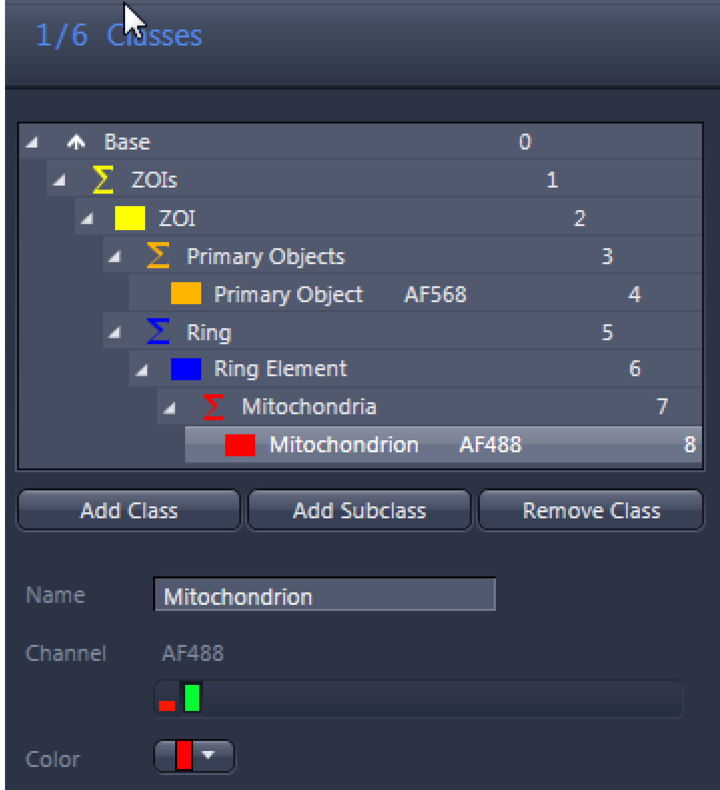
12.11.6.1 Step 1: Classes

Prerequisite

- ✓ You have created a new image analysis setting using the ZOI method.
- ✓ You have opened the **Image Analysis Wizard**. For more information, see *Creating a new image analysis setting [▸ 369]*.

1. In the **Image Analysis Wizard 1/6 Classes**, select Ring or Ring Element and click **Add Subclass** to extend the predefined set of classes with a subclass of the Ring Element.
2. Another class below the Ring Element is added. Give this class a meaningful name, e.g. Mitochondria / Mitochondrion.

3. The cell nuclei (primary objects) are stained with AF568 (red channel), therefore you need to select this channel to segment the cell nuclei. Select **Primary Object** and for **Channel** select **AF568**.



4. Click on **Mitochondrion** and in the field **Channel** select **AF488**.
   → The mitochondria are stained with **AF488**, therefore you need to use this channel for image segmentation.

12.11.6.2 Step 2: Frame

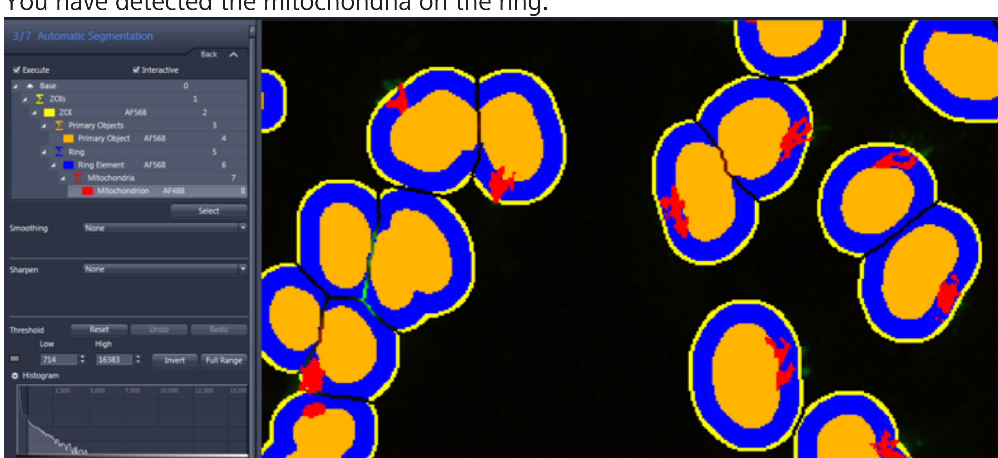
For more information, see *Step 2: Frame [▸ 384]*.

12.11.6.3 Step 3: Automatic Segmentation

Prerequisite ✓ For more information, see *Step 3: Automatic Segmentation [▸ 385]*.

1. Additionally, you need to set the segmentation parameters for the object **Mitochondrion**. Choose suitable parameters to segment the objects.

→ You have detected the mitochondria on the ring.



12.11.6.4 Step 4: Region Filter

For more information, see *Step 3: Automatic Segmentation [▸ 385]*.

See also

Measuring Mean Fluorescence Intensity on a Ring around the Primary Object [▸ 382]

12.11.6.5 Step 5: Features

You can define individual measurement features for each class. The measurement features defined for one class you can copy to the other classes via **Copy to all**.

1. Select the class for which you want to define measurement features, and click **Edit**. From the list of features on the right you can add features to the selected features list on the left.
   → These features are automatically defined for every object during image analysis. All classes have **ID of the parent** and **ID** as default features. This allows you to later to group the associated parameters from the excel lists, if necessary.
   → The class **Ring** additionally has **Area** and **Count** as default parameters.
2. In this example, we count the number of objects (the number of mitochondria fragments) within each ring. To attribute the number of mitochondria fragments to the **Primary Object**, select **Mitochondria** and click **Edit**.
   → The **Feature Selection** dialog opens.
3. In the parameter list on the right, select the feature **Count**. In the **Copy** drop-down menu, click .
   → This measurement feature is copied to the corresponding **Primary Object**.

12.11.6.6 Step 6: Preview Result

In this step you see a preliminary result of the image analysis.

1. Click on the different objects in the **Analysis** tab to get the preliminary measurement result for all objects.

2. Select the **Primary Object** to get a list with preliminary measurement results with the features ID of the parent, ID, Area, and Ring Mitochondria Counts. Note that the results are preliminary and only include the part of the image you see in the viewport.

	ID of the parent	ID	Area [µm²]	Ring Event...
	A	B	C	D
1	867	868	314.734	14
2	611	612	555.696	8
3	545	546	602.509	5
4	1,091	1,092	179.311	5
5	535	536	217.555	4
6	579	580	177.639	4
7	635	636	164.891	4
8	721	722	164.682	4
9	875	876	318.078	4
10	1,023	1,024	274.400	4
11	461	462	181.609	3
12	483	484	99.478	3
13	533	534	146.918	3
14	609	610	119.331	3
15	851	852	284.013	3
16	995	996	765.727	3
17	1,067	1,068	175.758	3
18	1,075	1,076	131.244	3
19	1,077	1,078	105.747	3
20	395	396	224.661	2
21	407	408	104.911	2
22	433	434	198.328	2
23	437	438	122.675	2
24	449	450	294.671	2
25	479	480	184.326	2

→ The result of the image analysis shows **Ring Mitochondria Counts** as a feature of **Primary Object**.

3. In the table, click on column **Ring Mitochondria Counts** to sort the entries in increasing or decreasing order.
4. Click on **Finish** to save the analysis settings and to close the wizard.

You can now run the analysis as described in *Measuring Mean Fluorescence Intensity on a Ring around the Primary Object [▸ 382]*.

12.11.7 Performing an interactive analysis

The image analysis tool allows you to perform an analysis interactively. It runs the selected analysis setting with all the steps that have been marked as interactive in the setup. Steps that you have not marked as interactive in the **Image Analysis Wizard** are run with the values predefined in the image analysis setting. The program does not pause to allow you to change these values interactively.

Analyze Interactively also allows you to directly execute an image analysis on an czi image without predefining an image analysis setting. For that you need to have an image analysis setting where all steps are marked as interactive. Then it is possible to modify every step of the **Image Analysis Wizard** during **Analyze Interactively** and do a one-time image analysis on the dataset without creating a new setting. In order to retrieve an image analysis setting from an already analyzed dataset, see *Creating an image analysis setting from an analyzed image [▸ 370]*.

Note: When you analyze an image interactively, the modifications of the settings during the interactive analysis are not saved.

Prerequisite ✓ You have defined an image analysis setting where all analysis steps you want to adjust interactively are marked as interactive.

1. On the **Analysis** tab, open the **Image Analysis** tool.
2. For **Setting**, select your image analysis setting.
3. Click on **Analyze Interactively**.

→ The **Image Analysis Wizard** opens with all the steps that are defined as interactive in the setting.

4. Modify your settings for each step and click on **Next** to get to the following step in the wizard.
5. At the end, click on **Finish** to close the wizard.

You have now analyzed your image interactively and the results of this analysis are displayed.

12.11.8 Creating a measurement data table

1. Click on the **Create Measurement Data Table** button on the **Analysis** tab.
   → The two data lists are now separate documents.
2. Save each of the data lists via the **File** menu | **Save As**. Allocate a name and select **.csv** as the file type.
   → The measurement data tables are saved in **CSV** format and can therefore be opened directly in Excel.
3. Click on the image and save it via the **File** menu | **Save As**. Allocate a name and select **.czi** as the file type.

The image is saved with the measurement results. If you open the image, the measurement results can be viewed in the **Analysis View**.

12.11.9 Charts and Tables of the Analysis View

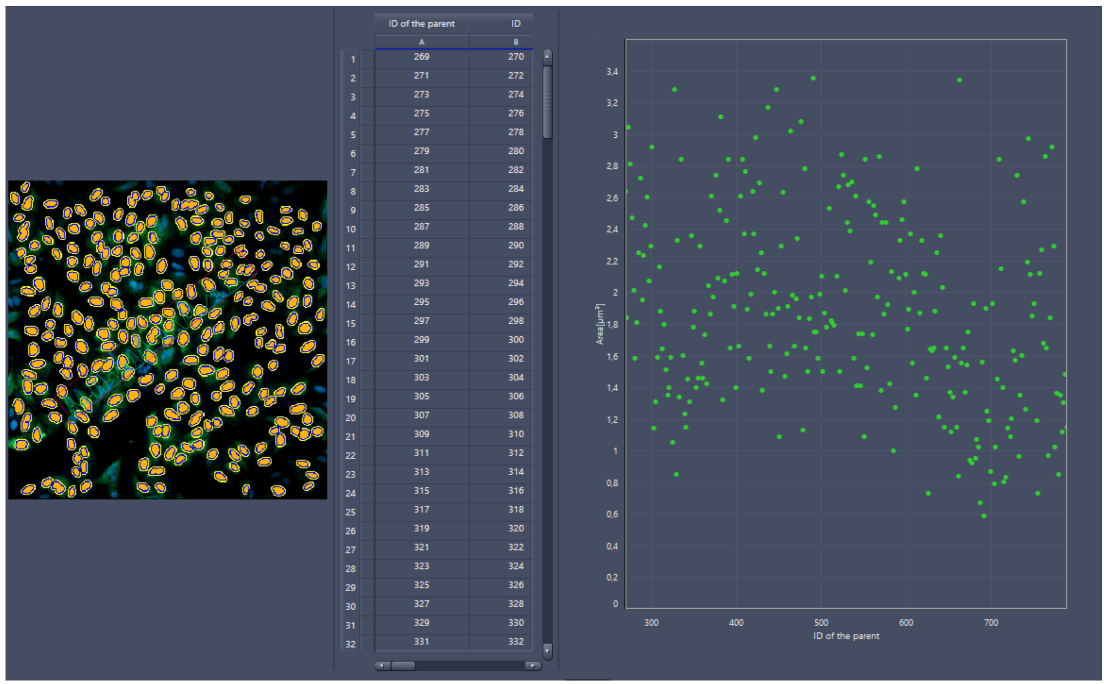
The **Analysis View** shows different charts and tables depending on the analysis, the experiment, and the selected options in the *Custom Chart tab [▸ 857]*. The axes of the charts are configured in the **Custom Chart** tab and the shown analysis results are always those of the class selected in the **Analysis** tab. An image of the charts can be exported with the *Chart Export Tab [▸ 856]*. You can zoom into a chart by using the mouse wheel or drawing a zoom rectangle. To zoom out to the original view, double click on the background of the plot.

Info

- To highlight the row of the table containing the measured values of an object, click on a segmented object in the image or in the chart. To highlight multiple rows, press *Ctrl* and click on multiple object/ data points.
- To highlight the corresponding segmented object in the image, click on a row in the table or on the data point in the chart. To highlight multiple objects, press *Ctrl* and click on multiple rows/ data points.
- To highlight the measured value of an object in the scatter chart or in the histogram, click on one or more rows in the table. The corresponding data point in the chart turns red. To change the chart type, on the **Custom Chart** tab, click on the corresponding **Chart Type** button. To highlight multiple objects, press *Ctrl* and click on multiple rows/ objects.

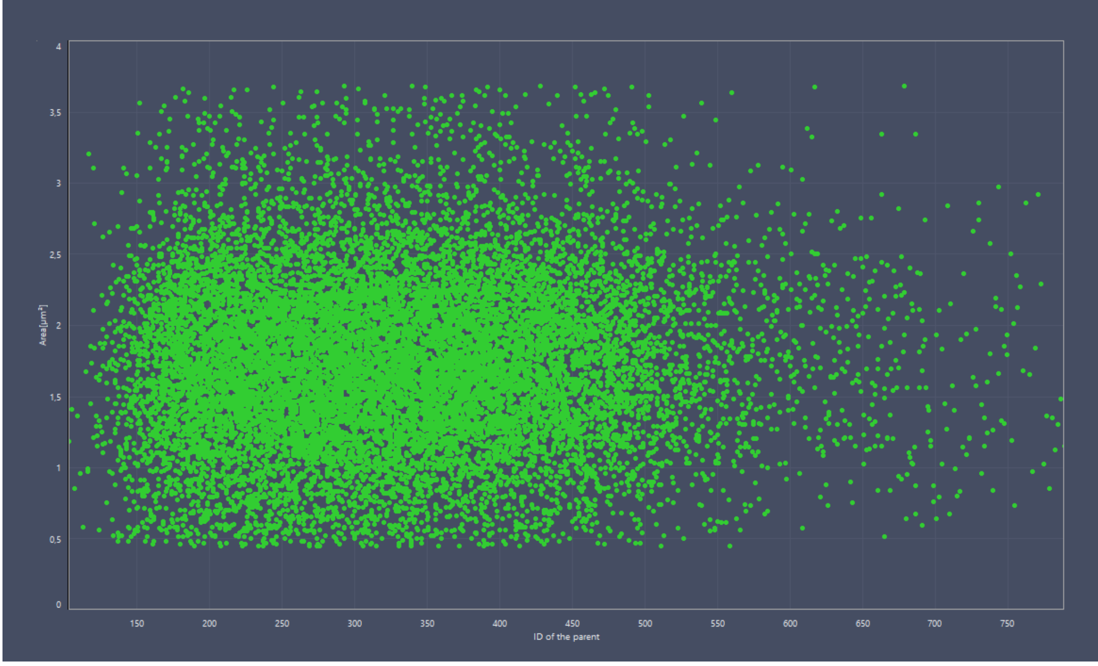
Standard table and chart

As a standard, a table with all analysis results and a chart configured according to the input in the **Custom Chart** tab are shown besides the analyzed image.



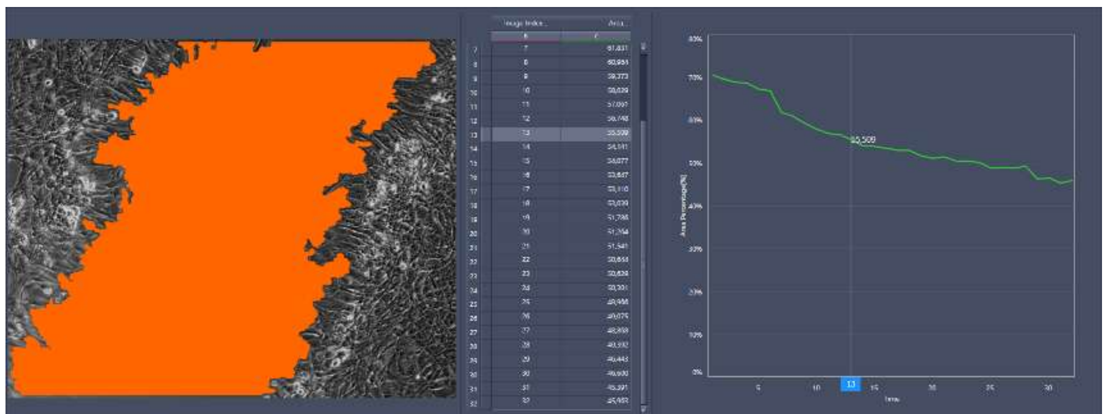
Multiple Scenes chart

For experiments containing multiple scenes, the **Analysis View** offers a chart which shows the results of all / multiple scenes. To see this chart, activate the checkbox **Multiple Scenes** in the **Custom Chart** tab. You can customize the displayed results with the **Sample Carrier** tab. For more information, see *Selecting data for the multiple scenes chart [▸ 396]*.



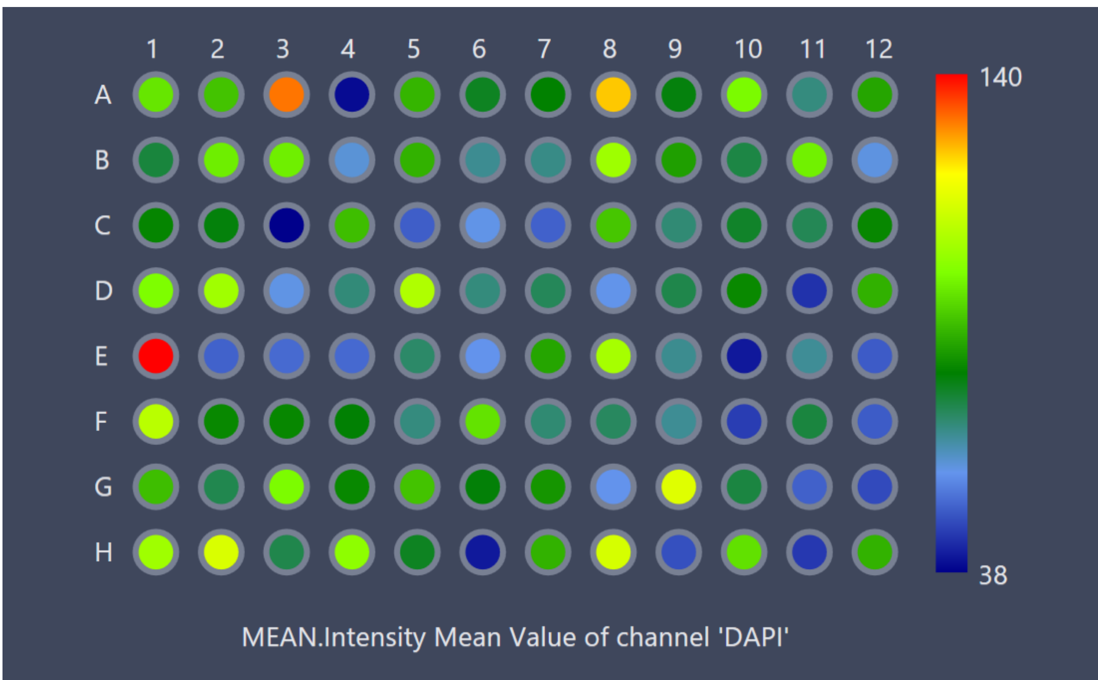
Time Series chart

For time series experiments, the **Analysis View** offers a chart and table which display the analysis results over time. To see this chart, activate the checkbox **Time Series** in the **Custom Chart** tab. You can use the slider (the blue box) to browse through the individual points in time, which highlights the respective data in the table, and vice versa.



Heatmap

For multi well / multi chamber experiments, the **Analysis View** offers a heatmap to display the measurement results on a well / chamber level. To see this map, activate the checkbox **Heatmap** in the **Custom Chart** tab.



See also

- Custom Chart tab [857]

12.11.9.1 Selecting data for the multiple scenes chart

Prerequisite ✓ In the **Custom Chart** tab, the **Multiple Scenes** checkbox must be activated.

1. Click on the **Sample Carrier** tab.
2. Select the desired well/ scene. To select multiple wells/ scenes, press the *Ctrl* key and click on the individual wells. Alternatively, select the desired wells with the pressed left mouse key. To select a specific region of wells, press the *Shift* key and click on the two wells which mark the corners of the region.

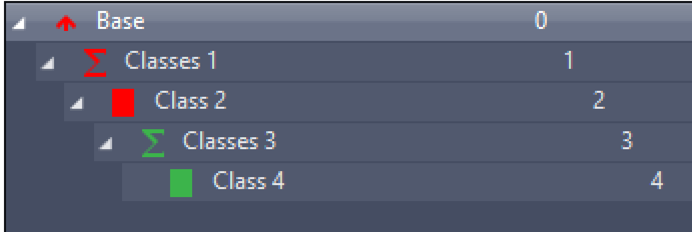
The chart displays the data of the selected wells/ scenes.

See also

- Custom Chart tab [▸ 857]
- Sample Carrier Tab [▸ 857]

12.11.9.2 Heatmap Calculation

The heatmap displayed in the Analysis View is calculated based on statistical features which you add in the Features step of the Image Analysis Wizard.
The statistical measurement is done on the basis of the top class objects of your analysis setting. Consider the following example:



To calculate, for example, the Mean Area of the **Classes 1**, the area of all **Class 2** objects is summed up and then the total area is divided by the count of objects.

This table shows the offered statistical parameters and the calculation description:

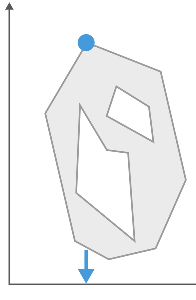
Parameter	Description
Area	Calculates the total area of all objects found in a well or chamber.
Area Percentage	Calculates the percentage that the area of all objects has in respect to the whole frame area (area of all objects divided by the whole frame area).
Count	Calculates the count of objects.
Intensity Std	Calculates the intensity standard deviation divided by the number of objects. $\frac{\sum_{s=1}^{s_n}\sum_{i=1}^{o_s} x_i}{\sum_{s=1}^{s_n} o_s}$ ▪ s_n = Number of the scenes in a well or chamber ▪ o_s = Number of objects found in the scene s ▪ x_i = Intensity standard deviation of the object i

Parameter	Description
Mean Intensity	Calculates the mean value of the mean of the intensity and divides it by the number all objects in all scenes. $$\frac{\sum_{s=1}^{s_n}\sum_{i=1}^{o_s} x_i}{\sum_{s=1}^{s_n} o_s}$$ ▪ s_n = Number of the scenes in a well or chamber ▪ o_s = Number of objects found in the scene s ▪ x_i = Intensity mean value of the object i
Mean Area	Calculates the mean value of the mean of the area and divides it by the number of all objects in all scenes. $$\frac{\sum_{s=1}^{s_n}\sum_{i=1}^{o_s} x_i}{\sum_{s=1}^{s_n} o_s}$$ ▪ s_n = Number of the scenes in a well or chamber ▪ o_s = Number of objects found in the scene s ▪ x_i = Mean area of the object i

12.11.10 Measurement Features

The software can automatically detect and measure the following properties of objects:

ACP X Unscaled

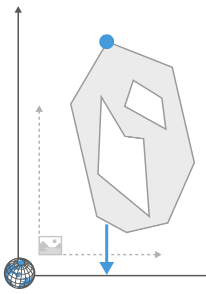


The x coordinate of the first pixel in the first line of a region

To identify measurement objects, the image is scanned from top left to bottom right. The so-called ACP (anti-coincidence point) is the first point that has been identified for a new object. The parameter **Acp X** indicates the x-coordinate of this point.

- Unit: pixels
- Value range: 1 ... image size in x-direction

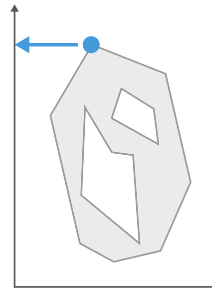
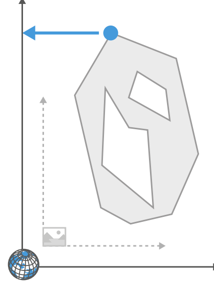
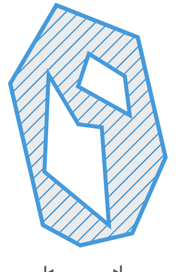
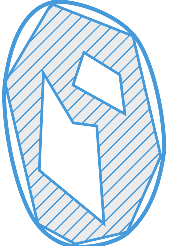
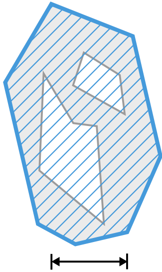
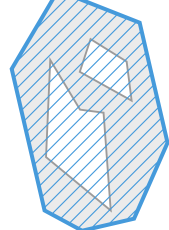
ACP X Unscaled WCS

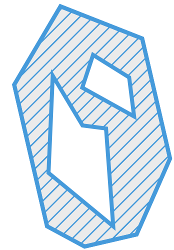
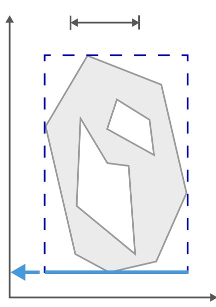
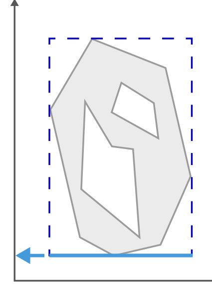
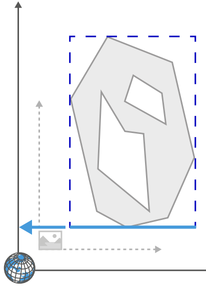
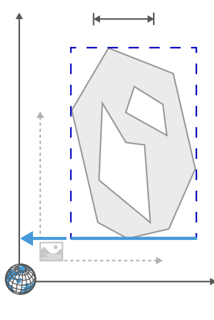
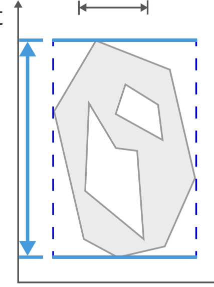


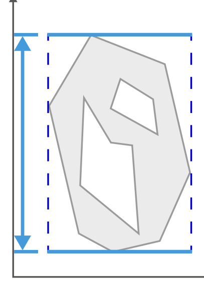
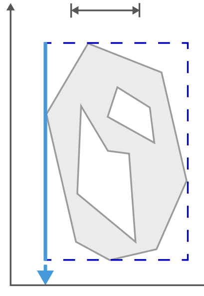
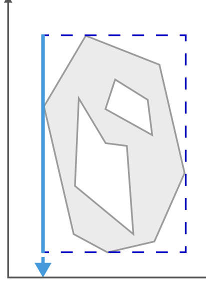
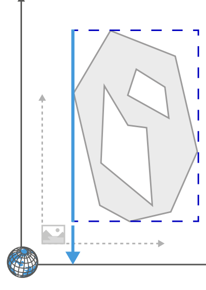
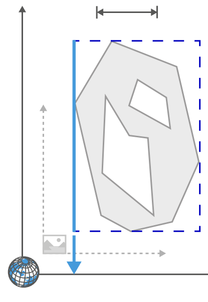
The x coordinate of the first pixel in the first line of a region

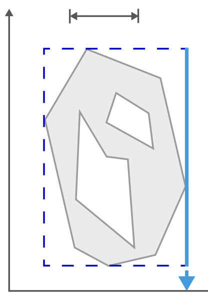
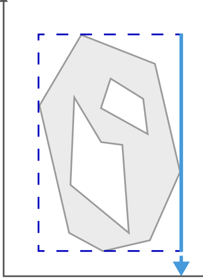
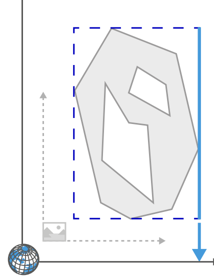
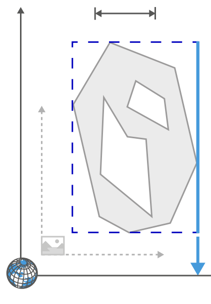
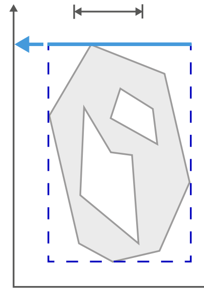
To identify measurement objects, the image is scanned from top left to bottom right. The so-called ACP (anti-coincidence point) is the first point that has been identified for a new object. The parameter **Acp X** indicates the x-coordinate of this point in the world coordinate system (WCS).

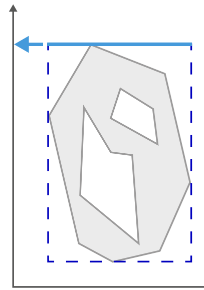
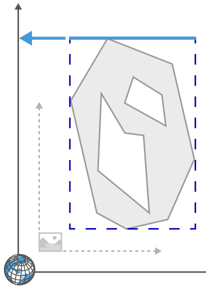
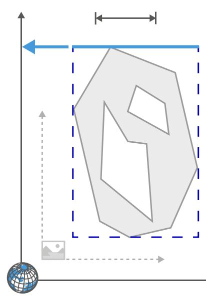
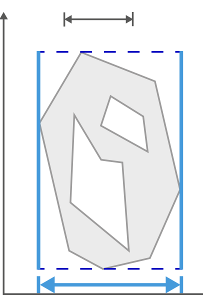
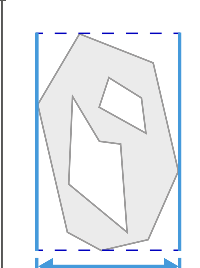
- Unit: pixels
- Value range: 1 ... image size in x-direction

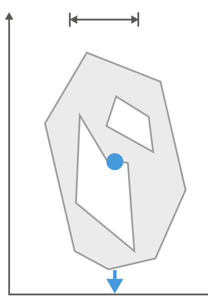
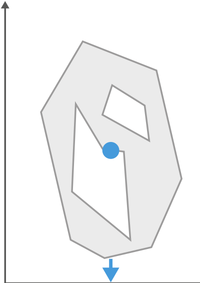
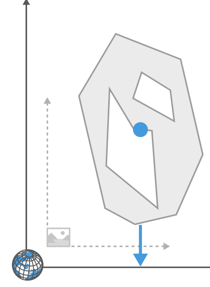
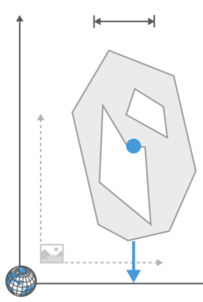
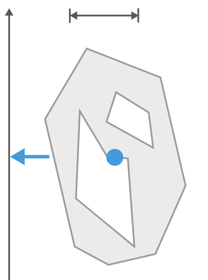
Parameter	Illustration	Description
ACP Y Unscaled		The y coordinate of the first pixel in the first line of a region To identify measurement objects, the image is scanned from top left to bottom right. The so-called ACP (anti-coincidence point) is the first point that has been identified for a new object. The parameter **Acp Y** indicates the y-coordinate of this point. ▪ Unit: pixels ▪ Value range: 1 ... image size in y-direction
ACP Y Unscaled WCS		The y coordinate of the first pixel in the first line of a region To identify measurement objects, the image is scanned from top left to bottom right. The so-called ACP (anti-coincidence point) is the first point that has been identified for a new object. The parameter **Acp Y** indicates the y-coordinate of this point in the world coordinate system (WCS). ▪ Unit: pixels ▪ Value range: 1 ... world coordinate size in y-direction
Area		Area of a region Area of a region excluding any holes it may contain. The areas of the holes are not included in the measurement. If you want to include them, use the **Area filled** parameter. ▪ Unit: Unit of area of the scaling assigned to the image (e.g. μm^2)
Area Convex		Area of convex hull of a region The current region is surrounded by a convex polyline. The (filled!) area of the resulting region is then measured. ▪ Unit: Unit of area of the scaling assigned to the image (e.g. μm^2)
Area Filled		Area of filled region Area of a region including any holes it contains. The holes are interpreted as belonging to the region or are filled prior to the measurement. If you do not want the holes to be measured, use the **Area** parameter. ▪ Unit: Unit of area of the scaling assigned to the image (e.g. μm^2)
Area Filled Unscaled		Area of filled region Area of a region including any holes it contains. The holes are interpreted as belonging to the region or are filled prior to the measurement. If you do not want the holes to be measured, use the **Area** parameter. ▪ Unit: $pixels^2$

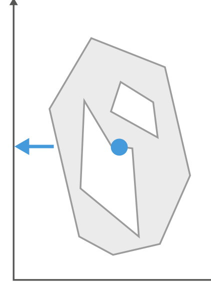
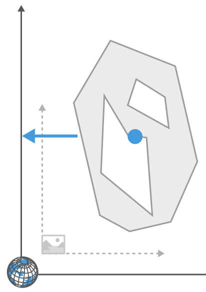
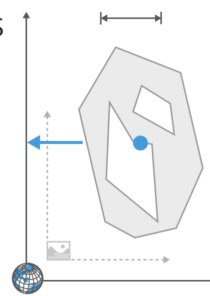
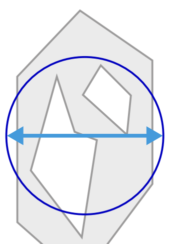
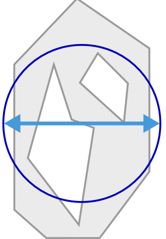
Area Un-scaled		Area of a region unscaled The **Area unscaled** parameter corresponds to the **Area** parameter. However, the scaling of the image is not taken into account for the measurement. The (unfilled!) area of a region is displayed in pixels in each case. ▪ Unit: pixels²
Bound Bot-tom		Minimum y-coordinate of the bounding box of a region Indicates the y-coordinate of the bottom edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Bound Bot-tom Unscaled		Minimum y-coordinate of the bounding box of a region Indicates the y-coordinate of the bottom edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: pixels
Bound Bot-tom Unscaled WCS		Minimum y-coordinate of the bounding box of a region Indicates the y-coordinate in the world coordinate system (WCS) of the bottom edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: pixels
Bound Bot-tom WCS		Minimum y-coordinate of the bounding box of a region Indicates the y-coordinate in the world coordinate system (WCS) of the bottom edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Bound Height		Indicates the height (size in y-direction) of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm) ▪ Formula: Bound top - Bound bottom

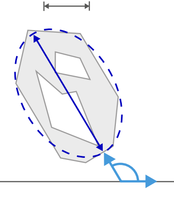
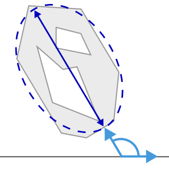
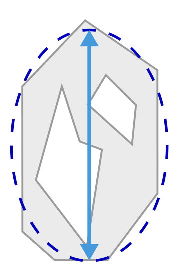
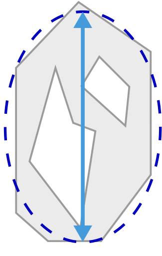
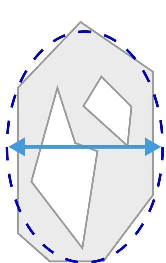
Bound Height Unscaled		Indicates the height (size in y-direction) of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: pixels ▪ Formula: Bound top - Bound bottom
Bound Left		Minimum x-coordinate of the bounding box of a region Indicates the x coordinate of the left-hand edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Bound Left Unscaled		Minimum x-coordinate of the bounding box of a region Indicates the x coordinate of the left-hand edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: pixels
Bound Left Unscaled WCS		Minimum x-coordinate of the bounding box of a region Indicates the x coordinate in the world coordinate system (WCS) of the left-hand edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: pixels
Bound Left WCS		Minimum x-coordinate of the bounding box of a region Indicates the x coordinate in the world coordinate system (WCS) of the left-hand edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)

Bound Right		Maximum x-coordinate of the bounding box of a region Indicates the x coordinate of the right-hand edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Bound Right Unscaled		Maximum x-coordinate of the bounding box of a region Indicates the x coordinate of the right-hand edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: pixels
Bound Right Unscaled WCS		Maximum x-coordinate of the bounding box of a region Indicates the x coordinate in the world coordinate system (WCS) of the right-hand edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: pixels
Bound Right WCS		Maximum x-coordinate of the bounding box of a region Indicates the x coordinate in the world coordinate system (WCS) of the right-hand edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Bound Top		Maximum y-coordinate of the bounding box of a region Indicates the y coordinate of the top edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)

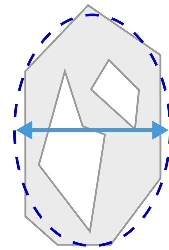
Bound Top Unscaled		Maximum y-coordinate of the bounding box of a region Indicates the y coordinate of the top edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: pixels
Bound Top Unscaled WCS		Maximum y-coordinate of the bounding box of a region Indicates the y coordinate in the world coordinate system (WCS) of the top edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: pixels
Bound Top WCS		Maximum y-coordinate of the bounding box of a region Indicates the y coordinate in the world coordinate system (WCS) of the top edge of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Bound Width		Indicates the width (size in x-direction) of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Formula: Bound right - Bound left ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Bound Width Unscaled		Indicates the width (size in x-direction) of a bounding box for a region. The box is drawn in parallel to the x and y axis. ▪ Formula: Bound right - Bound left ▪ Unit: pixels

Center X		The x coordinate of the geometric center of gravity of a region Depending on the shape of the object, this point may also lie outside a region. The associated y-coordinate is determined via the **Center Y** parameter. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Center X Un-scaled		The x coordinate of the geometric center of gravity of a region Depending on the shape of the object, this point may also lie outside a region. The associated y-coordinate is determined via the **Center Y** parameter. ▪ Unit: pixels
Center X Un-scaled WCS		The x coordinate in the world coordinate system (WCS) of the geometric center of gravity of a region Depending on the shape of the object, this point may also lie outside a region. The associated y-coordinate is determined via the **Center Y** parameter. ▪ Unit: pixels
Center X WCS		The x coordinate in the world coordinate system (WCS) of the geometric center of gravity of a region Depending on the shape of the object, this point may also lie outside a region. The associated y-coordinate is determined via the **Center Y** parameter. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Center Y		The y coordinate of the geometric center of gravity of a region Depending on the shape of the object, this point may also lie outside a region. The associated y-coordinate is determined via the **Center X** parameter. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)

Center Y Unscaled		The y coordinate of the geometric center of gravity of a region Depending on the shape of the object, this point may also lie outside a region. The associated y-coordinate is determined via the **Center X** parameter. ▪ Unit: pixels
Center Y Unscaled WCS		The y coordinate in the world coordinate system (WCS) of the geometric center of gravity of a region Depending on the shape of the object, this point may also lie outside a region. The associated y-coordinate is determined via the **Center X** parameter. ▪ Unit: pixels
Center Y WCS		The y coordinate in the world coordinate system (WCS) of the geometric center of gravity of a region Depending on the shape of the object, this point may also lie outside a region. The associated y-coordinate is determined via the **Center X** parameter. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Circularity		Sqrt(Roundness) = Sqrt(4 × Area / (π × FeretMax²))
Compactness		4 × π × Area / PerimeterConvex²
Convexity		PerimeterConvex / PerimeterCrofton
Diameter		Diameter of a circle with an area equal to that of the object The object is measured using the **Area**) parameter. A circle with the same area as the object is created. The diameter of this circle is returned. ▪ Formula: Sqrt ((4 / π) × Area) ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Diameter Unscaled		Diameter of a circle with an area equal to that of the object The object is measured using the **Area**) parameter. A circle with the same area as the object is created. The diameter of this circle is returned. ▪ Formula: Sqrt ((4 / π) × Area1) ▪ Unit: pixels

Ellipse Angle		Angle of the major axis of the ellipse The major axis of an ellipse with the same geometric moment of inertia as the current region is determined in accordance with the **Ellipse major** parameter. The angle to the x-axis is then determined. The indication of the angle always relates to a counterclockwise direction. ▪ Unit: degrees ▪ Value range: 0 ... 180°
Ellipse Angle Unscaled		Angle of the major axis of the ellipse The major axis of an ellipse with the same geometric moment of inertia as the current region is determined in accordance with the **Ellipse major** parameter. The angle to the x-axis is then determined. The indication of the angle always relates to a counterclockwise direction. ▪ Unit: degrees ▪ Value range: 0 ... 180° This tool uses unscaled pixels for calculating the angle. The results may differ from the results of Ellipse Angle.
Ellipse Major		Length of the major axis of the ellipse Length of the major axis of an ellipse with the same geometric moment of inertia as the region. The moment of inertia is calculated about the center of gravity of the region. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Ellipse Major Unscaled		Length of the major axis of the ellipse Length of the major axis of an ellipse with the same geometric moment of inertia as the region. The moment of inertia is calculated about the center of gravity of the region. ▪ Unit: pixels
Ellipse Minor		Length of the minor axis of the ellipse Length of the minor axis of an ellipse with the same geometric moment of inertia as the region. The moment of inertia is calculated about the center of gravity of the region. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)

Ellipse Minor Unscaled

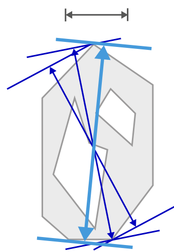


Length of the minor axis of the ellipse

Length of the minor axis of an ellipse with the same geometric moment of inertia as the region. The moment of inertia is calculated about the center of gravity of the region.

- Unit: pixels

Feret Maximum

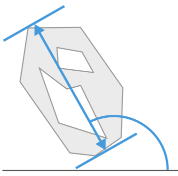


Maximum feret of a region

The maximum feret of a region is determined on the basis of distance measurements. Two straight lines are positioned on opposite sides of the object, like a sliding caliper, at 128 angle positions. The corresponding distance is measured for each angle position. The maximum value determined is the maximum feret.

- Unit: Unit of the scaling assigned to the image (e.g. µm)

Feret Maximum Angle

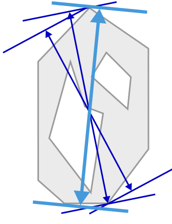


Angle of the maximum feret of a region in relation to the x-axis

The maximum feret is determined as described in **Feret Maximum**. The angle of the maximum feret in relation to the x-axis is then determined. The indication of the angle always relates to a counterclockwise direction.

- Unit: degrees
- Value range: 0 ... 180°

Feret Maximum Unscaled

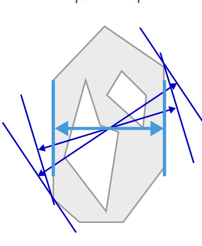


Maximum feret of a region

The maximum feret of a region is determined on the basis of distance measurements. Two straight lines are positioned on opposite sides of the object, like a sliding caliper, at 128 angle positions. The corresponding distance is measured for each angle position. The maximum value determined is the maximum feret.

- Unit: pixels

Feret Minimum

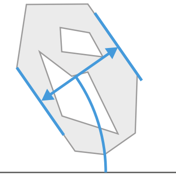


Minimum feret of a region

The minimum feret of a region is determined on the basis of distance measurements. Two straight lines are positioned on opposite sides of the object, like a sliding caliper, at 128 angle positions. The corresponding distance is measured for each angle position. The minimum value determined is the minimum feret.

- Unit: Unit of the scaling assigned to the image (e.g. µm)

Feret Minimum Angle

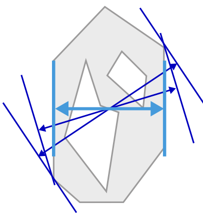


Angle of the minimum feret of a region in relation to the x-axis

The minimum feret is determined as described in **Feret Minimum**. The angle of the minimum feret in relation to the x-axis is then determined. The indication of the angle always relates to a counterclockwise direction.

- Unit: degrees
- Value range: 0 ... 180°

Feret Minimum Unscaled

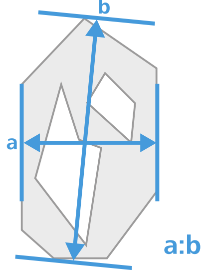


Minimum feret of a region

The minimum feret of a region is determined on the basis of distance measurements. Two straight lines are positioned on opposite sides of the object, like a sliding caliper, at 128 angle positions. The corresponding distance is measured for each angle position. The minimum value determined is the minimum feret.

- Unit: pixels

Feret Ratio

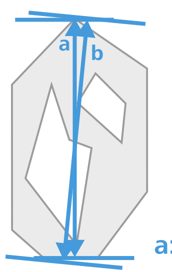


Feret ratio

The ratio of **Feret Minimum** to **Feret Maximum** is calculated. This ratio makes it possible to make statements on the form of the measured objects. If the feret ratio has a low value, long, elongated objects are present. Values approaching 1 indicate the presence of compact or circular objects, as in this case **Feret Minimum** and **Feret Maximum** have very similar values. The **Form circle** is also suitable for making statements on the circularity of an object.

- Formula: FeretMin / FeretMax (a / b)
- Unit: none
- Value range: 0 ... 1

Feret Vertical to Maximum



The ratio between the feret distance at 0° and the **Feret Maximum**

- Unit: none

Fiber Length

Length of a fiber-like region

To calculate the fiber length, a structure that is actually similar to a fiber is required. Here it is not the distance between a start and end point that is determined. The check can be done using the **Form circle**, among other things.

- Formula: ¼ × (Perimeter + (Sqrt (Perimeter^2 - 16 × Area))
- Unit: Unit of the scaling assigned to the image (e.g. µm)

Fiber Length Unscaled

Length of a fiber-like region

To calculate the fiber length, a structure that is actually similar to a fiber is required. Here it is not the distance between a start and end point that is determined. The check can be done using the **Form circle**, among other things.

- Formula: ¼ × (Perimeter1 + (Sqrt (Perimeter1^2 - 16 × Area))
- Unit: pixels

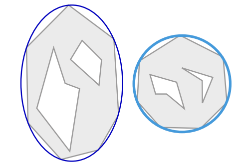
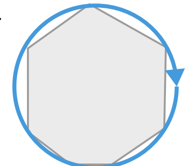
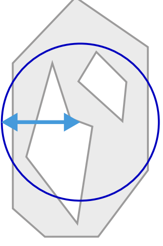
Form Circle		Form factor of a region Describes the form of a region on the basis of its circularity. A perfect circle is given the value 1. The more elongated the region is, the smaller the form factor. The calculation is based on the **Area filled** and **Perimeter Crofton** parameters. ▪ Formula: $4 \times \pi \times$ Area / PerimeterCrofton^2 ▪ Unit: none ▪ Value range: 0...1
ID		Sequential ID of the object
ID of the Parent		Sequential ID of the object's parent
Image Container Category		Returns the container category (e.g. sample, control) of the current scene (well).
Image Index Block		Index for the individual image slices of a multi-block image. Multi-block images are composed of blocks of different dimensions.
Image Index Position		Index for the individual image slices of a **Position List** image. If the image was acquired using the **Position List** tool, the **Image Index Position** returns the number of the image that contains the object. In contrast, the **ID** parameter is a global counter, i.e. the number of the object. The **Index Position** can be used to guarantee unambiguous assignment between measured regions in the image and the individual lines of a data table, especially in cases where several images are measured, as automatic assignment is then no longer possible. To achieve this, the **Index Region** parameter must also be selected as a region feature and also be inserted into the graphics plane as an annotation(Draw Features). It is also advisable to activate the **Image name** parameter as a region feature so that the correct original image can be reloaded. ▪ Restriction: This value is only available for images that have previously been acquired with AxioVision or saved in AxioVision ZVI format.

Image Index Scene	Index for the individual image slices of a scene image Indicates the unambiguous number of the scene in an image. The word **Scene** refers to a coherent object on a slide that contains several objects for examination. The **Index Scene** can be used to guarantee unambiguous assignment between measured regions in the image and the individual lines of a data table, especially in cases where several images are measured, as automatic assignment is then no longer possible. To achieve this, the **Index Region** parameter must also be selected as a region parameter and must also be inserted into the graphics plane as an annotation (Draw Features). It is also advisable to activate the **Image name** parameter as a region parameter so that the correct original image can be reloaded.
Image Index Time	Index for the individual image slices of a time series image.
Image Index Z	Index for the individual image slices of a Z stack image.
Image Scene Name	Returns the name of the scene (well).
Index	
Intensity Maximum of channel 'C1'	The pixel value of the brightest pixel in the object.
Intensity Mean Value of channel 'C1'	The average brightness (pixel value) of the pixels in the object.
Intensity Minimum of channel 'C1'	The pixel value of the darkest pixel in the object.
Intensity Range of channel 'C1'	The difference between the pixel value of the brightest and darkest pixels in the object, i.e. **Intensity Maximum of channel 'C1'-Intensity Minimum of channel 'C1'**
Intensity Standard Deviation of channel 'C1'	The standard deviation of the brightness (pixel value) of the pixels in the object.
Number of the region holes	Calculates the number of holes enclosed by the object

Perimeter		Perimeter of a region This parameter is specially optimized for measuring the perimeters of circles. If the measured region contains holes, the total perimeter including the perimeters of the hole structures is determined. If you only want the perimeter of the outside contour to be determined, use the **Perimeter filled** parameter. ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Perimeter Unscaled		Perimeter of a region This parameter is specially optimized for measuring the perimeters of circles. If the measured region contains holes, the total perimeter including the perimeters of the hole structures is determined. If you only want the perimeter of the outside contour to be determined, use the **Perimeter filled** parameter. ▪ Unit: pixels
Radius		Radius of a circle with an area equal to that of the object The object is measured using the **Area**) parameter. A circle with the same area as the object is created. The radius of this circle is returned. ▪ Formula: Sqrt ((1 / π) × Area) ▪ Unit: Unit of the scaling assigned to the image (e.g. µm)
Radius Unscaled		Radius of a circle with an area equal to that of the object The object is measured using the **Area**) parameter. A circle with the same area as the object is created. The radius of this circle is returned. ▪ Formula: Sqrt ((1 / π) × Area1) ▪ Unit: pixels
Region Class Color		Returns the color of the class to which the object is assigned
Region Class ID		Returns the ID of the class to which the object is assigned
Region Class Name		Returns the name of the class to which the object is assigned
Roundness		Formula: 4 × Area / (π × FeretMax^2)

12.12 ImageJ

This extension offers following possibilities:

- Easy exchange of images, from simple two-dimensional images, to more complex, multidimensional entities, like Z-stacks, time series and so on. The exchange can go both ways, from ZEN to ImageJ, as well as from ImageJ to ZEN.
- Execute functions in ImageJ, without having to leave the ZEN environment.
- Combine the two benefits, introduced above: sending a ZEN image to ImageJ, having it processed there, and then returning the resulting image back to ZEN in one single step.

Note that different versions and variants of ImageJ and Fiji exist. This document is based on the ImageJ/Fiji version 1.46. See notes for specifics of other versions and variants. For the sake of simplicity, Fiji is implied also, wherever ImageJ is mentioned in the following text.

12.12.1 Preparations

Info

Note that the extension for ImageJ is not available in **ZEN lite**.

1. Install **ImageJ** on your computer. Make sure that you use the latest version (check for online updates after installation).
2. Download **loci_tools.jar** and drop it into in the **ImageJ/plugins** folder.
3. Note the name of the folder with your preferred alternative. While you can switch freely among them all, it makes sense to stick to one and the same environment, once you have started to add your own programs and macros.
4. The ImageJ/Fiji folder you will eventually decide on, can either belong to you alone or be shared among other users of the system. It is up to you decide, what you prefer: if you are the only user, nobody will meddle with its contents (images, macros etc), but then, you will need to copy and distribute the contents, if they are of interest to others as well.

You have successfully fulfilled all prerequisites. You can now continue with setting up ImageJ within ZEN software.

12.12.2 Activate ImageJ Extension

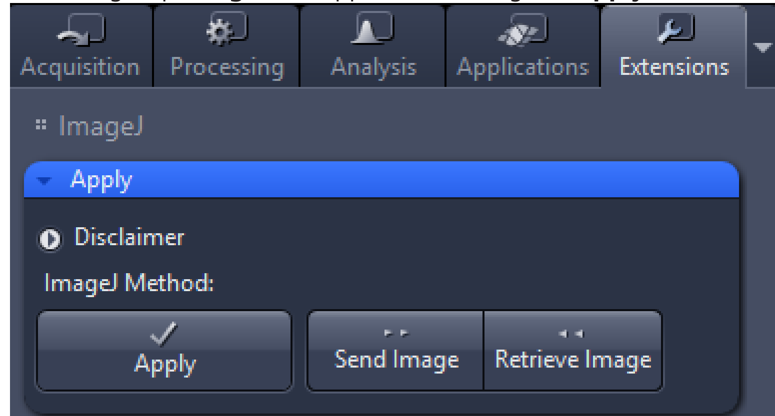
The extension is automatically included in the ZEN installation. To set it up, start the software and then proceed as follows:

1. Go to **Tools > Options > ImageJ**.
2. In the **ImageJ Executable** dropdown list, select your preferred **ImageJ.exe**.
3. If you prefer another executable or your preferred executable is not in the list, click on the last entry **...** to search for it.

You have successfully set up ImageJ extension within the software. Now you can start working with the extension.

12.12.3 Sending and retrieving images

1. In the **Left Tool Area**, click on the **Extensions** tab.
   → The tool group **ImageJ** will appear containing the **Apply** tool.



2. To send an image from **ZEN** to **ImageJ** select the image in ZEN.
3. In the **Apply** tool, click on the **Send Image** button.
   → The image will be sent to ImageJ and opened within a new image frame. You can now edit the image within the ImageJ application.
4. To retrieve an image from **ImageJ** to **ZEN** select the image in ImageJ.
5. Click on the **Retrieve Image** button.
   → The image will be opened in ZEN.

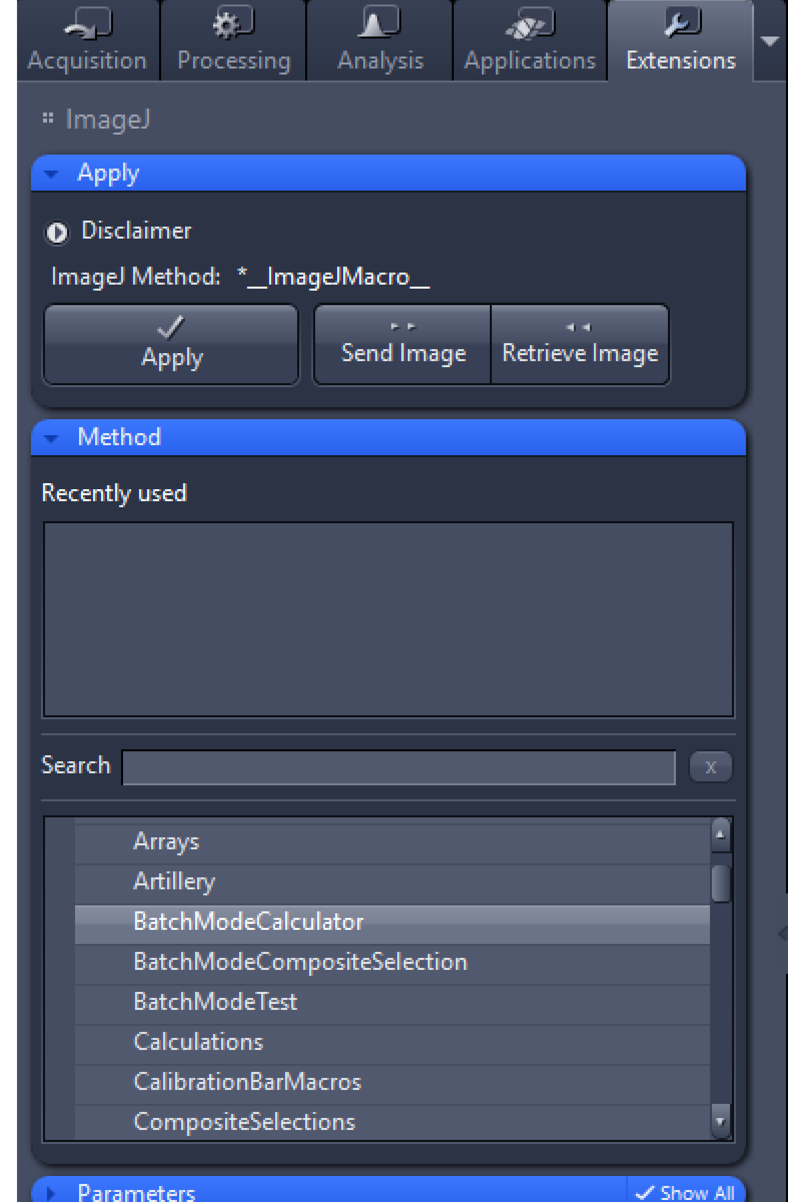
You have successfully exchanged images between ZEN and ImageJ.

12.12.4 Use ImageJ Methods

The extension offers the possibility of sending images to ImageJ to get processed, to retrieve the result of the operation or both. The following instruction will show the basic steps which are necessary to apply ImageJ methods on any images.

Prerequisite ✓ You are on the **Extensions** tab in the **ImageJ** group.

1. In the **Methods** tool select the command or method to be executed, e.g. an ImageJ macro.



2. In the **Parameters** tool, specify if the method selected will need an input image and/or provide a resulting image.
3. In the **Apply** tool, click on the **Apply** button to execute the command.

You have successfully applied an method to an image.

12.12.5 Image Type Send/Retrieve Conventions

ZEN to ImageJ

Image Type	Received as…	Comments
.tif, .jpg, .bmp, .png, .gif	Original	
.ome.tif	Original	

Image Type	Received as…	Comments
2D image B/W .czi	32-bit (RGB)	Convert the image in **ImageJ** to the required pixel type using Image > Type command
2D image 24/48 bit color .czi	32-bit (RGB)	
2D image 36/42 bit color .czi	-	Convert the CZI image to 24/48 bits before sending it or using it in a method
Multi-channel x Z-Stack x T-series	MD image	If necessary reassign the dimensions using Image > Hyperstacks for instance. Channel colors may be different from those set in ZEN
Tiled images	-	Only the first tile gets loaded.
12bit B/W images	Error in ImageJ	Workaround: convert the pixel type of the image to 16 bits in ZEN

ImageJ to ZEN

Image Type	Received as…	Comments
.tif, .jpg, .bmp, .png, .gif	Original	
.ome.tif	Original	
2-D images, B/W and RGB	B/W, RGB TIF	
Multi-channel x Z-Stack x T-series	MD image	Hint: select RGB in Quick Color Setup to get the same colors for channels as in ImageJ
Tiled images	-	Only the first tile gets loaded.

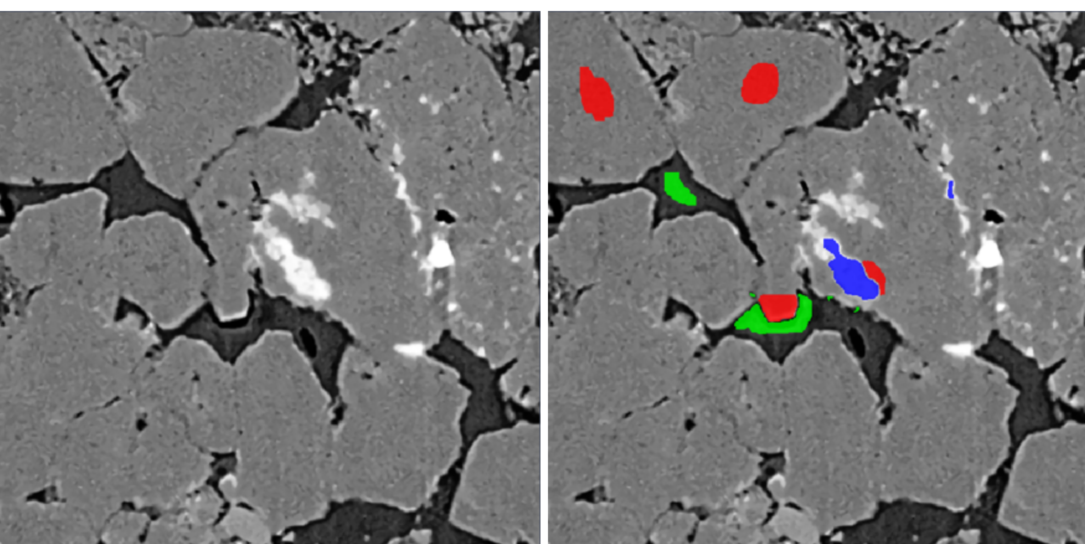
12.13 Intellesis

This module enables you to use machine-learning algorithms for segmenting images using pixel-classification. It uses different feature extractors to classify pixels inside an image based on the training data and the labeling provided by the user. There are a variety of use cases because the functionality itself is "data-agnostic" meaning it can be used basically with every kind of image data.

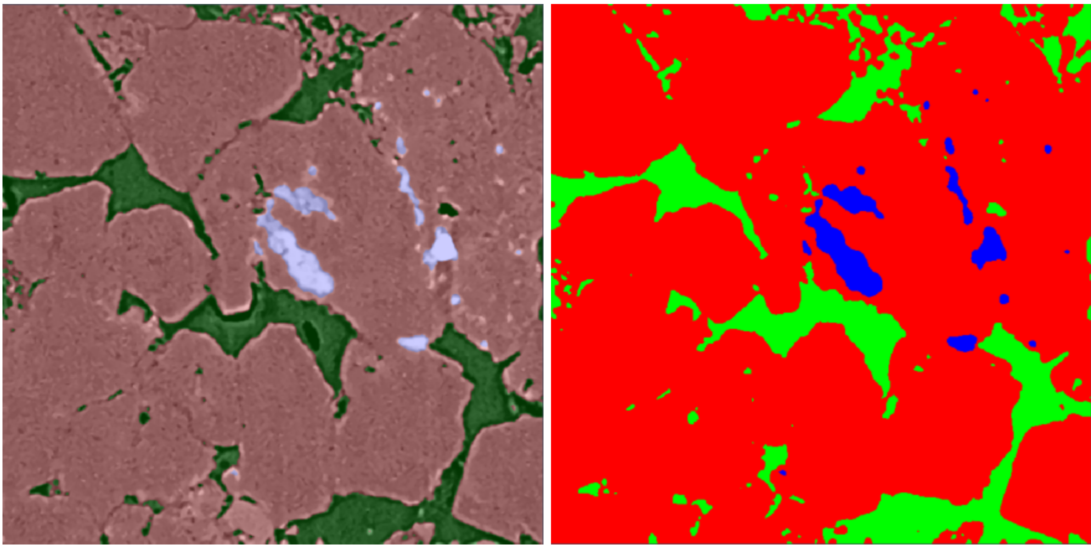
The extension has the following main functionality:

- Any user can intuitively perform image segmentation without advanced training by simply labeling what shall be segmented.
- Import of any image format readable by the software, incl. **CZI**, **OME-TIFF**, **TIFF**, **JPG**, **PNG** and **TXM** (special import required).
- Creation of predefined image analysis settings (*.czias) using machine-learning based segmentation that can be used inside the ZEN measurement framework.
- Integration of the Trainable Segmentation processing function within the OAD environment.

Application Example: XRM (X-Ray Microscopy) image from sand stone showing the main steps when working with the **Intellesis Trainable Segmentation** module.



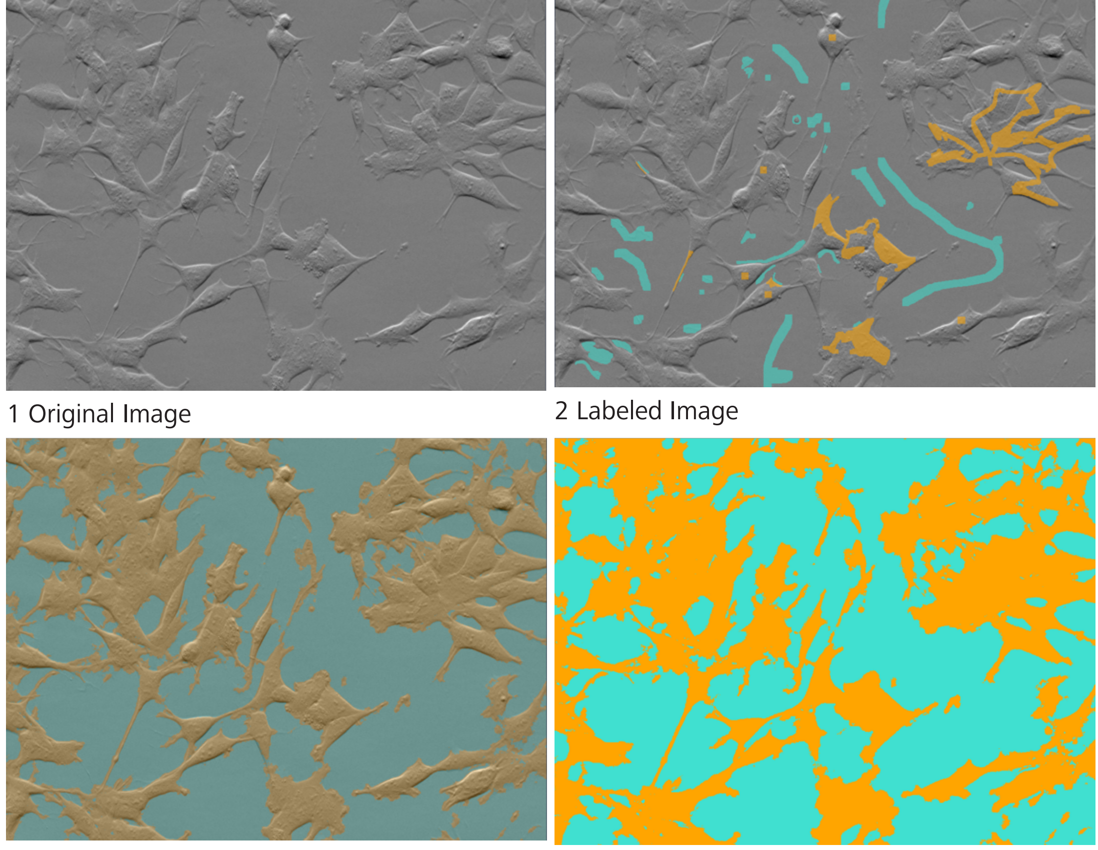
1 Original Image 2 Labeled Image



3 Overlay of Original Image and Segmentation Result

4 Segmented Image

Application Example: Cells image with Phase Gradient Contrast on the Celldiscoverer 7 and segmented using **Intellesis Trainable Segmentation**.



1 Original Image

2 Labeled Image

3 Overlay of Original Image and Segmentation Result

4 Segmented Image

Note:
The training of **Intellesis** models is CPU / GPU specific. A model trained on GPU only runs on a GPU machine. If a model trained on GPU is transferred to a CPU-only machine, the model has to be retrained to run on this machine.

12.13.1 Fact Sheet

- **Simple User Interface for Labelling and Training**
- **Integration into ZEN Measurement Framework**
- **Support for multi-dimensional datasets**
- Machine-Learning Tool for Pixel Classification powered by Python
  - Scikit-Learn
  - Tensorflow
  - Dask
- Client / Server Architecture with REST-API
- Engineered Default Feature Sets (CPU)
  - 25 or 33 Features
- Neural Network (vgg19) Layers for Feature Extraction (GPU)
  - 64, 128 (red. 50) or 256 (red. 70) Features for 1st, 2nd or 3rd layer
- Random Forest Classifier for **Pixel Classification (CPU)**
- Option to download pre-trained DNNs (Deep neural Networks) for specific sample types (subject of change)
- Post Processing: Conditional Random Field (CRF)
- IP-Functions for creating masks and confidence maps
- Python Scripting Integration able for advanced automation
- SW Trial License available

12.13.2 FAQ/Terminology

Term / Question	Description
Machine Learning	The **Intellesis Trainable Segmentation** module uses machine learning to automatically identify objects within an image according to a predefined set of rules (the model). This enables any microscopy user to perform image segmentation even on complex data sets without programming experience or advanced knowledge on how to set up an image segmentation.
What is a "Model" ?	A model is a collection of rules according to which the software attributes the pixels to a class. Such a class is mutually exclusive for a given pixel, i.e. a pixel can only belong to one class. The model is the result of (repeated) labeling and training a subset of the data. After the model is trained (the labels provided by the user were used to "train" the classifier), it can be applied to the full data set in image processing or it can be used to create an image analysis setting (*.**czias**) to be used with the ZEN image analysis module. In image processing the trained model can be applied to an image/or data set and perform segmentation automatically. As result you will get two images, the segmented image on the one hand and a confidence map on the other hand.

Term / Question	Description
What is a "Class" ?	A class is a group of objects (consisting of individual pixels) with similar features. According to the selected model the pixels of the image will be attributed as belonging to a certain class, e.g. cell nuclei, inclusions in metals, etc.. Every model has by default already two classes built-in, because at least two classes are needed (e.g. cells and background or steel and inclusions). Of course, more classes can be defined if necessary.
What is "Labeling" ?	Instead of using a series of complex image processing steps in order to extract the features of the image, you just need to label some objects in the image that belong to the same class. Based on this manual labeling the software will attribute the pixels of the image as belonging to a certain class. In order to refine the result, you can re-label wrongly attributed pixels and this way assign them to another class.
What is "Training" ?	During the training process (within the training user interface) you can repeatedly label structures as belonging to one class, run the training, check if the result matches your expectation and if necessary refine the labeling in order to improve the result. The result is a trained model (a set of rules) which produce the desired result when applied to the training data. With the labeled pixels and their classes a classifier will be trained. The classifier will then try to automatically assign single pixels to classes.
Training UI (User Interface)	The user interface for training is the starting point of the automatic image segmentation process. Here you import images, label and train the model which you can later use for automatic image segmentation. Within this interface you can load the training data, define the classes of objects found in your data and train the classifier to assign the objects to the correct classes.
What is "Segmenting" or "Segmentation"?	In general segmentation is the combination of pixels of the same class within an image. Before you can perform segmentation the segmentation model has to be trained. Within the Training UI you train the software by labeling specific objects or structures that belong to different classes. A pseudo-segmentation is performed each time you train the model so that you see if the feature extractor works for your image. One output of the **Intellesis Trainable Segmentation** processing function is the fully segmented image or data set using the trained model. The second output is the confidence map.
Confidence Map	The confidence map is one of two resulting images when you apply a trained model to an image by using the processing function **Intellesis Trainable Segmentation**. The (resulting) grayscale image encodes the reliability of the segmentation. Areas which can be addressed to a certain class with a high confidence will appear bright, whereas areas which have a lower confidence to belong to a certain class will appear dark. The confidence is represented by a percentage value, where 0 means "Not confident at all" (dark) and 100 "Very confident" (bright).
What is a "Feature"?	A feature is a specific property of an image, that will be calculated by using a predefined set of filters and processing functions. This process results a so-called "Feature Vector" for every pixel. This is the information that will be used for training the model.

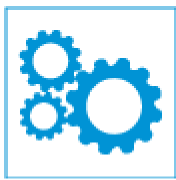
Term / Question	Description
What is a "Feature Extractor"?	A feature extractor is a predefined set of processing functions that is used to create the feature vector for every pixel. As feature extractor a specific layer of a pre-trained neuronal network can be used as well.
Prediction	When the model that was trained on example data is applied to a new unlabeled data set the result is called a prediction.
Multi-Channel Images	The **Intellesis Trainable Segmentation** module supports multi-channel data sets. It is important to understand that in case of multi-channel images every pixel can still only belong to one class, i. e. the classes are mutually exclusive. The additional information of having more than one intensity value per pixel (e.g. one for every channel) is also used for classification. **Example**: If you have overlapping regions A and B in the image that you want to classify then consider labeling three independent classes: ▪ Class 1: A ▪ Class 2: B ▪ Class 3: A overlapping with B If you want to segment an individual channel from a multi-channel image, use the **Create Subset** IP function first to extract the desired channel.

12.13.3 Operating Concept

The operating concept can be generally split in three parts:

Training

The **Training User Interface** which is accessed via the **Intellesis Trainable Segmentation** tool on **Analysis** tab. Within the training user interface you can label the images to be used as input for training a specific model, see *User Interface - Training [▸ 421]*.



Processing

The **Image Processing** (IP) function **Intellesis Trainable Segmentation**, which can be used to segment images resulting in binary masks. Those masks can be used in subsequent ZEN workflows, such as 2D or 3D analytics or they can be exported for further use in external 3rd party software packages. You will find more details under *Using a Trained Model for Image Processing [▸ 434]*.

Analyzing

The automatic creation of **Image Analysis** (IA) settings (*.**czias**), which allows to use a trained model for automated segmentation and measurement of image data within the ZEN **Image Analysis Wizard**. To familiarize with the basic steps take a look at our step-by-step guide in the chapter *Using a Trained Model for Image Analysis [▸ 435]*.

12.13.4 Workflow Overview

ZEN Intellesis Trainable Segmentation offers three main workflows. The general workflows and the basic steps involved are shown inside the diagram.

- Labeling and Training your images -> results in a **Trained Model**.
- Using the Trained Model to segment images -> results in **Binary Masks**.
- Using the Trained Model for image analysis -> results in classified pixel for subsequent segmentation and measurements of objects.

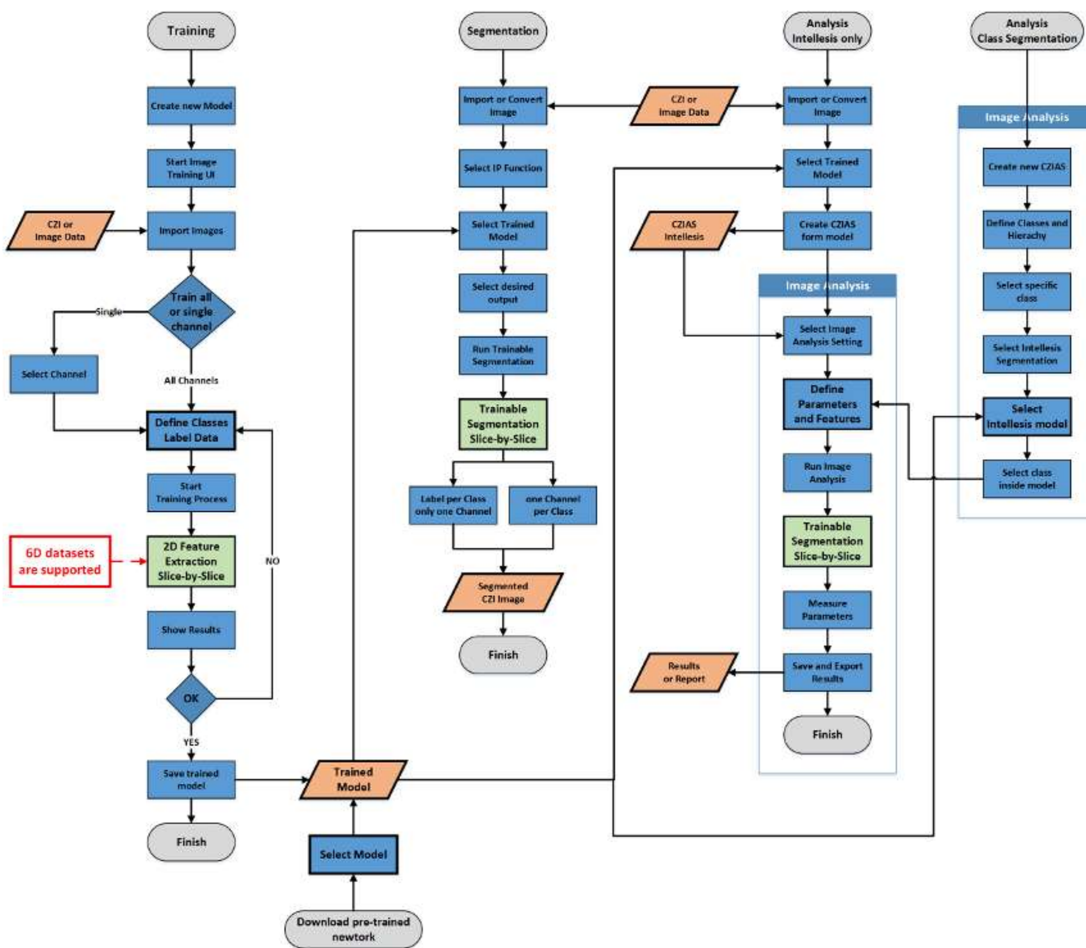


Fig. 23: Process description of the Intellesis workflow

12.13.5 User Interface - Training

The Training user interface is accessed via the **Analysis** tab. Open the Intellesis **Trainable Segmentation** tool, select or create a new a model and click on **Start Image Training**. The user interface for training will be visible:

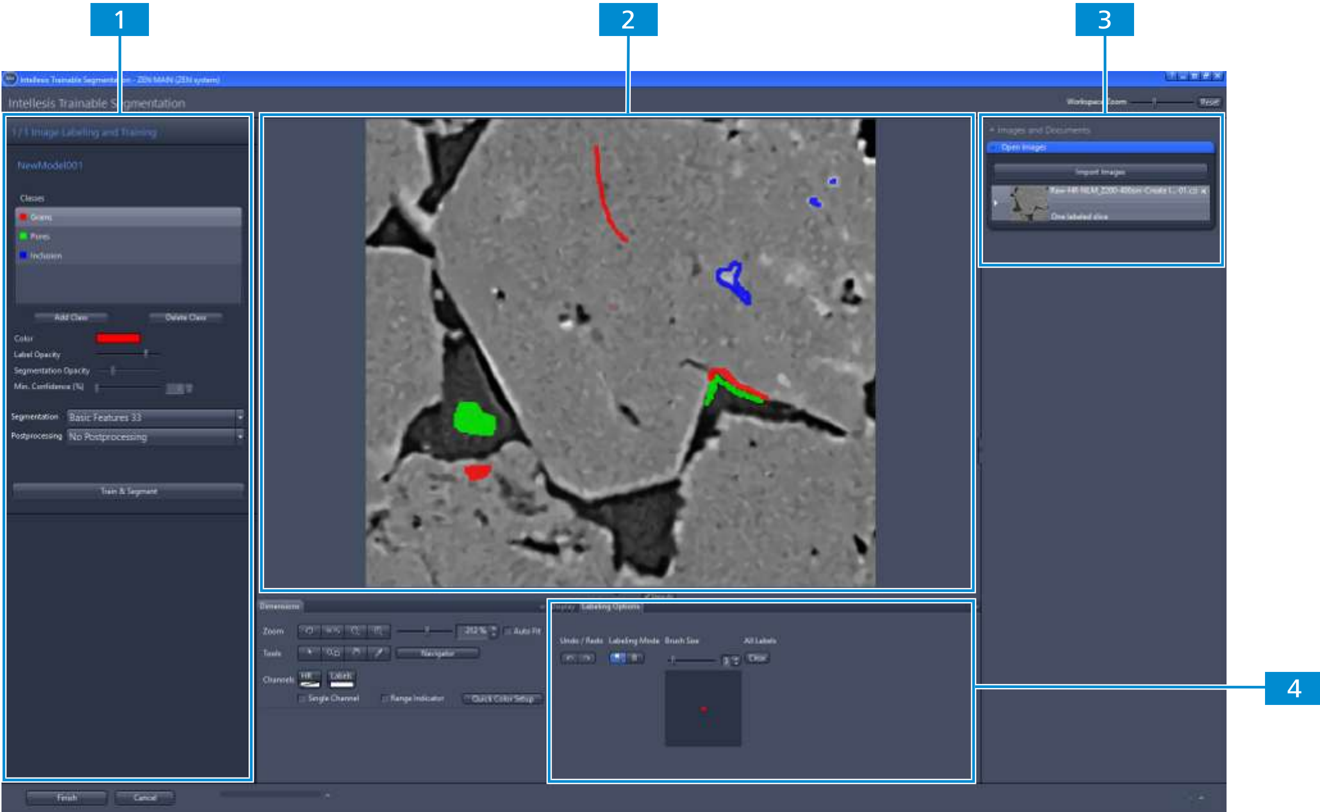


Fig. 24: User Interface for Training

1 Labeling and Training Settings
On the left side you find elements for managing the classes. You can add and delete classes and select them for labeling an image.
You can change the label opacity and the segmentation opacity by adjusting the corresponding slider. Opacity determines to what degree it obscures or reveals labels or segmentations. Opacity of 1% appears nearly transparent, whereas 100% opacity appears completely opaque. Additionally, you can hide all segmented pixels where the confidence value is below a certain threshold set by the **Min. Confidence (%)** slider.
With the different parameters of the **Segmentation** options and the **Postprocessing** options it is possible to further improve the results of the training and the (pseudo-)segmentation. The **Train & Segment** button starts the automatic training algorithm and then performs the pseudo-segmentation of the defined classes in the image.

2 Center Screen Area
This area shows the sample image. You can directly start labeling the classes within the image.
When you are inside the image, the actual brush size for labeling is represented by a square. If the brush size is very small, the square is changed into a dotted circle with a small point inside.

3 Image Gallery
On the right side you can import and select the images you want to use for training and segmenting.

4 Labeling Options
Below the center screen area you can adjust the **Labeling Mode** or **Brush Size**.

Info

When you use images with large X/Y dimensions, e.g. large tile images, the segmentation will be only performed on a subset of the whole image in order to avoid long waiting periods. The current image subset maximum size in X/Y is 5000 pixels and is centered on the current view port. Nevertheless all labels inside the complete image will be used for training, but the segmentation preview (pseudo-segmentation) will be only applied to that subset.

12.13.5.1 Segmentation Options

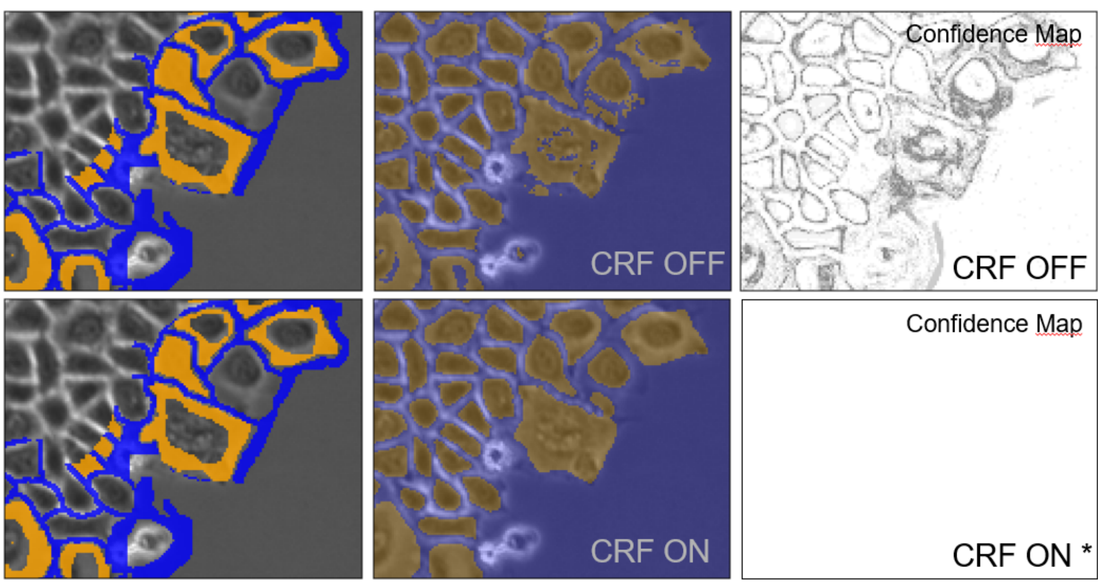
Parameter	Description
Basic Features 25	A predefined feature set using 25 features.
Basic Features 33	A predefined feature set using 33 features.
Deep Features 50 **Deep Features 64** **Deep Features 70** **Deep Features 128** **Deep Features 256**	The complete or reduced feature set from either the 1st, 2nd or 3rd layer of a pre-trained network is used to extract the respective number of features.

For the selection of the parameters note the following:

- There is no "right" selection. We recommend to always try different parameters for the same image to see which one works best.

12.13.5.2 Postprocessing Options

Parameter	Description
No Postprocessing	This parameter is set by default. No further postprocessing will be applied on the images.
Conditional Random Field (CRF)	If selected, this post processing function is applied to the output of the pixel classification. This can improve the segmentation results, depending on your sample. The CRF algorithm tries to create smoother and shaper borders between objects by re-classifying pixels based on confidence levels in their neighborhood. Note: If CRF is activated, the returned confidence map does not reflect the outcome of the majority votes of all decision trees of a specific class anymore. Therefore, a map containing only ones will be returned when the CRF postprocessing option is activated.



12.13.5.3 Labeling Options

Parameter	Description
Undo / Redo	When you click on the arrows you can undo/redo the last actions you have performed.
Labeling Mode	Here you can select between labeling and erase mode.
Brush Size	Here you can set the brush size of the labeling / erasing tool. Note that the brush size can be changed alternatively by holding the *Strg* key and using the mouse wheel (when the cursor is inside the image area.)
All Labels	When you click on **Clear**, all labels in the active image will be deleted.

12.13.5.4 Image Gallery

In the right tool area under **Images & Documents** you find the area for handling the images to be used for training. Here you can load and select the images you want to use for training. When you click on a loaded image, the image will be visible in the **Center Screen Area**.

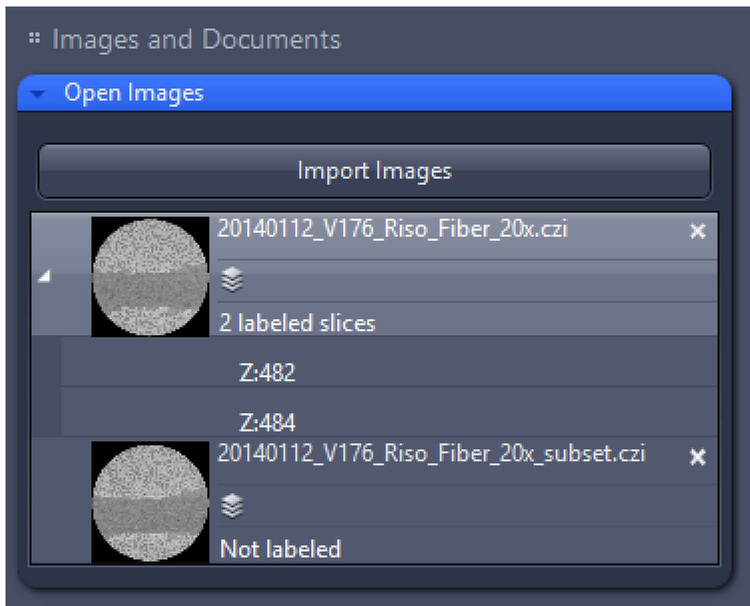


Fig. 25: Image Gallery with additional info

In the list of images you have certain possibilities to gain advanced information about the image. If you load a new image only the preview image, file name and type of image are displayed.

When you have started to label an image of a larger data set, a small arrow appears on the left side of the preview image. If you click on the arrow, a list of the images that contain labels will be displayed, containing dimension and image number (e.g. for a Z-stack, Z:400 indicates that the slice number 400 contains labels).

If you click on this information the corresponding image will be automatically displayed in the center screen area. This is very helpful when you are working with large data sets such as z-stacks, scenes or time-series and you want to quickly load the image which you have already labeled.

12.13.5.5 Select Channel dialog

This dialog is only displayed if a multi-channel image is imported.

Parameter	Description
Training Mode	Here you can select the mode for training.
– Single Channel	Only one channel of the image is imported for training. Such models trained on one channel can only be used inside the **Image Analysis Wizard** for the **Intellesis Class Segmenter**.
– Multispectral	All channels of the image are imported for training.
Select Channel	Only visible if **Single Channel** is chosen as the training mode. In the drop down list you can choose the channel you want to import for training.

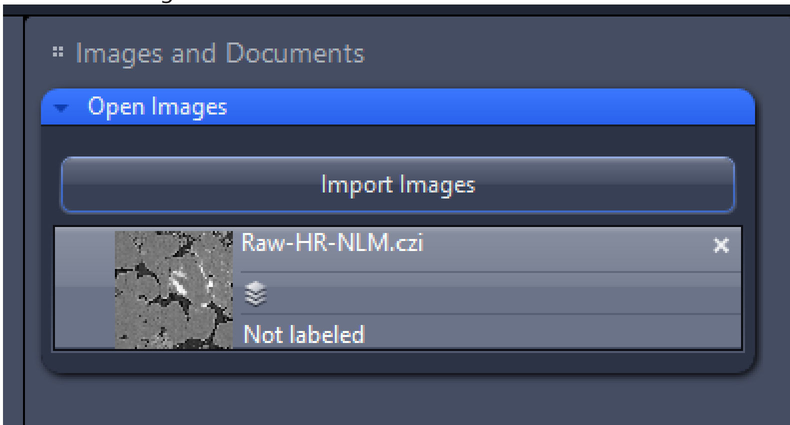
Parameter	Description
OK	Confirms the settings and imports the image accordingly.
Cancel	Cancels the image import and closes the dialog.

12.13.6 Intellesis Models

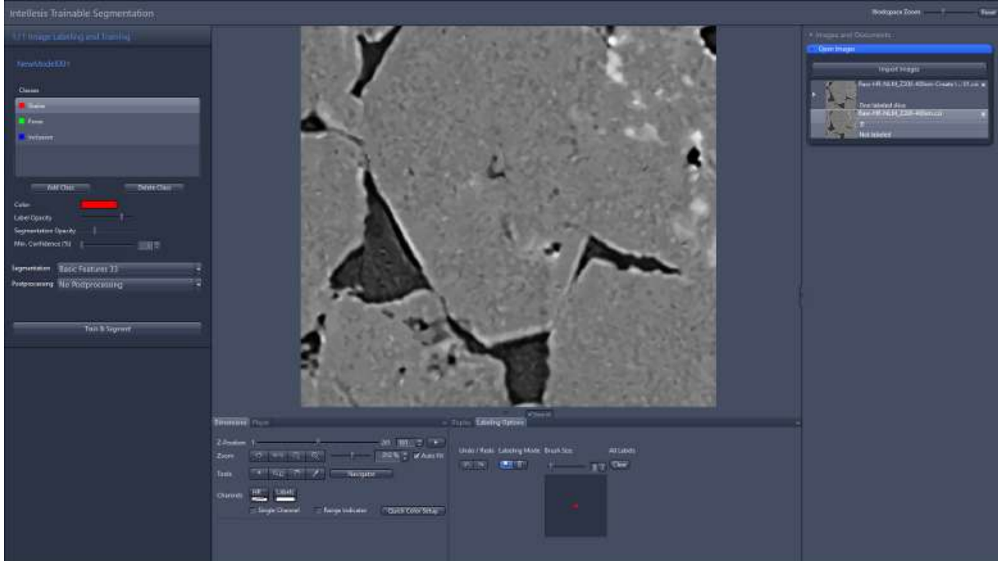
12.13.6.1 Creating a New Model

Prerequisite ✓ You have completed the general preparations.

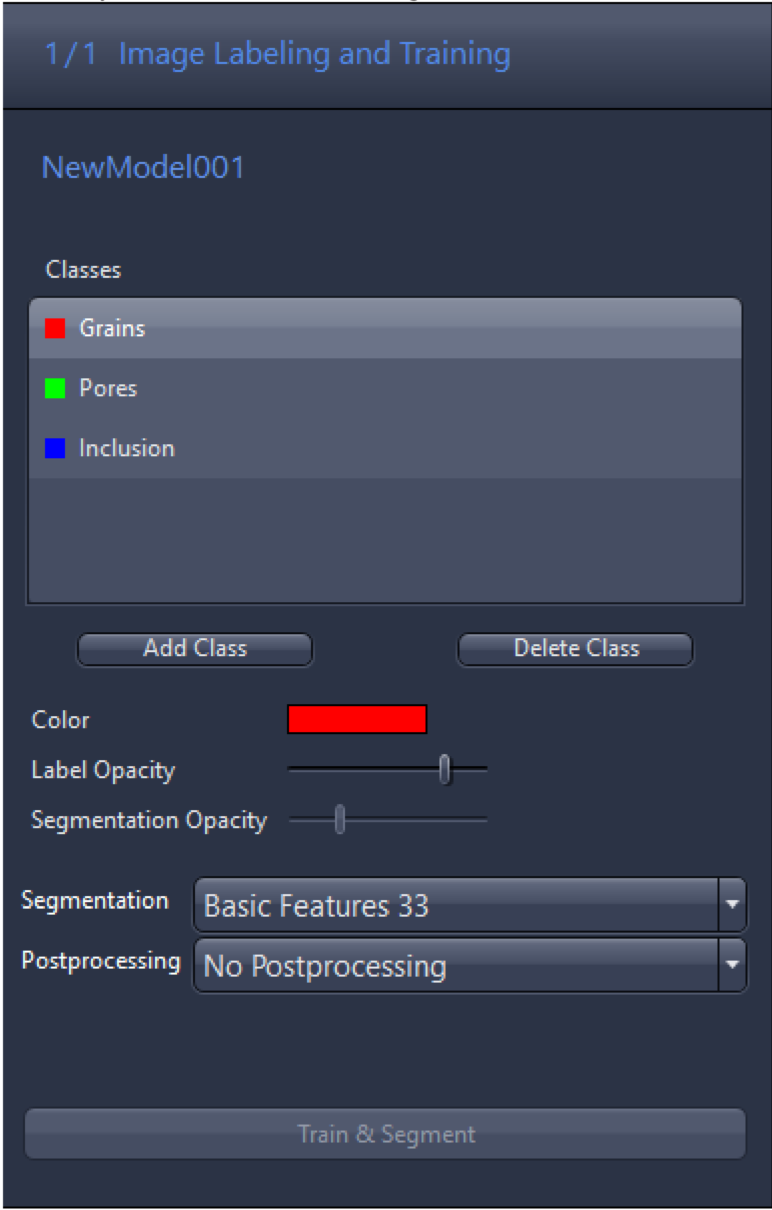
1. On the **Analysis** tab, in the **Intellesis Trainable Segmentation** tool, click on **Options** and select **New**.
2. Enter a name and a description for the new model and click on
3. Click on **Start Training**.
   → The user interface for training opens.
4. In the **Right Tool Area** under **Open Images** click on **Import Images**.
5. Select the image for training from the file system and click on **Open**.
   → The image will be visible in the list. Note that all imported images will be included to your training model.
6. Select the image from the list.



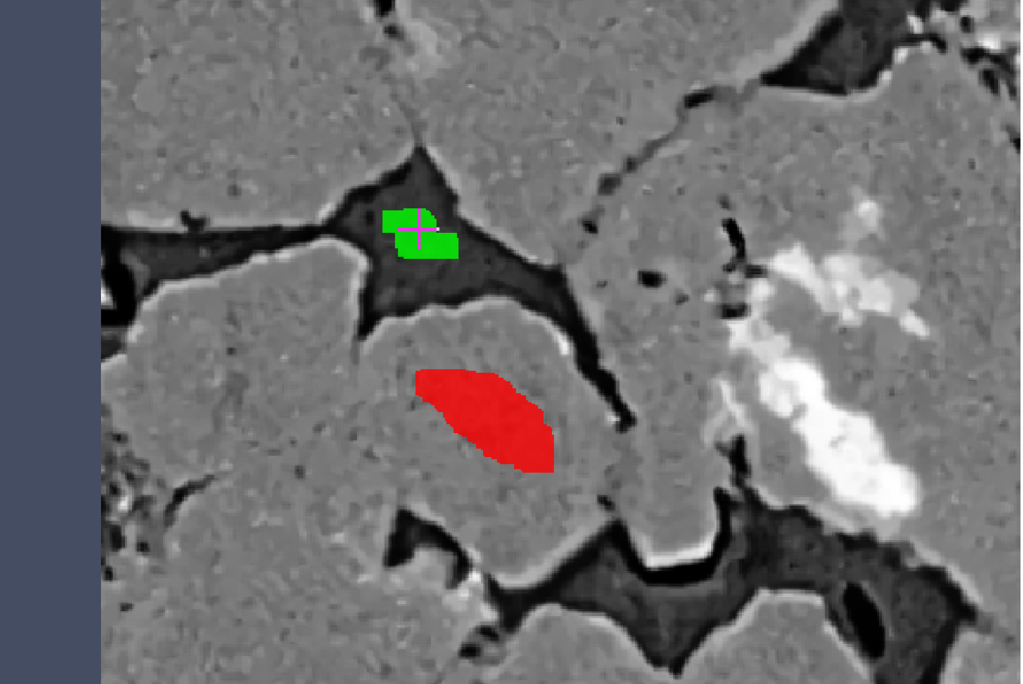
→ The image will be displayed in the **Center Screen Area**. Note that on a later stage you can add more images to refine the training via **Import Images**.



7. Switch to the **Left Tool Area** and continue with the definition of the classes. Depending on your image and what you want to segment you can define a certain amount of classes. When you start with a new model you will see the two predefined classes **Object** and **Background**. If you click on **Add Class** a new class is added. You can rename these classes by a double-click and entering a new name.



8. Now move the courser inside the image and start labeling the areas which you want to assign to the selected class. To label within the image simply hold down the left mouse key and move the mouse.



9. After labeling a few areas with different classes click on **Train & Segment**.
   → The software will now start the training. The system will try to automatically recognize other areas of the same classes. Depending on the image, the pixel classification can take a while. When finished the image will have the additional channel **Seg**(menation) containing the segmentation preview.
10. If you are not satisfied with the result you can label more details of the corresponding classes. Therefore you can zoom into the image or change the brush size of the courser. The more accurate you label the different classes within the image, the better the recognition will be. When you finished the labeling you have to click on **Train & Segment** again. You can repeat that process until you are satisfied with the segmentation result.
    Note that at this point as a result you will only see a pseudo segmented image and only the area visible in the main window will be segmented (max. area 5000x5000 px). The full segmentation of an image/data set is performed on the **Processing** tab by using the trained model within the **Trainable Segmentation** processing function.

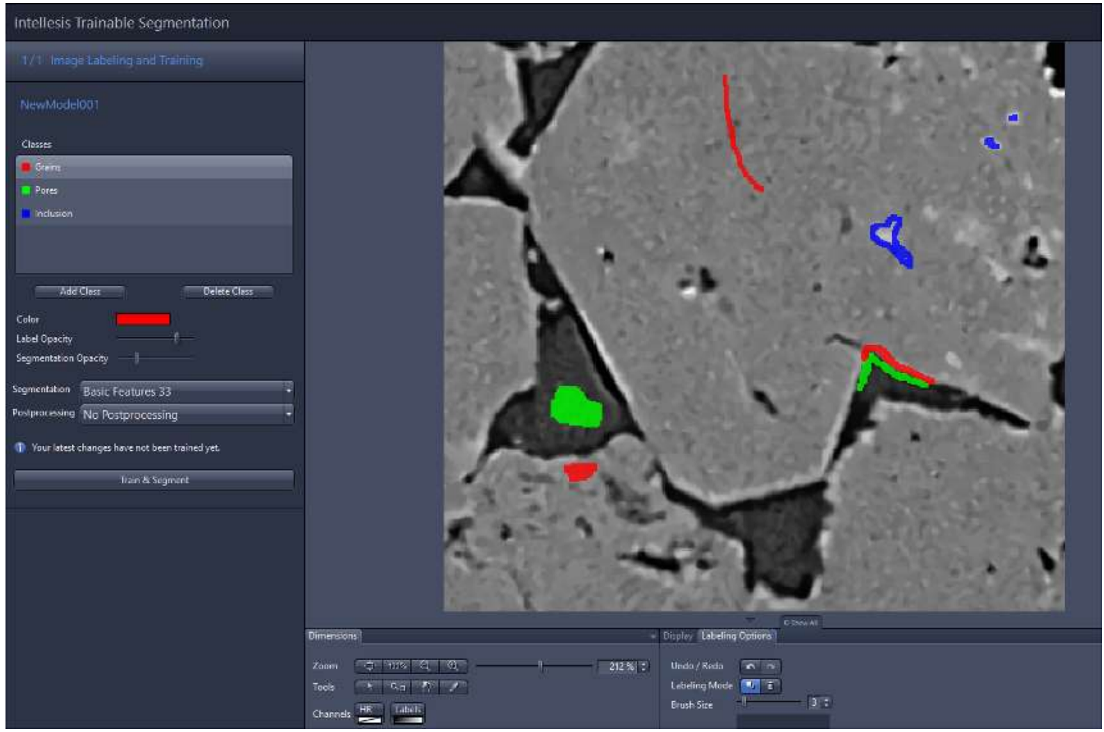


Fig. 26: Trained model with refined labeling and pseudo segmentation results

Info

For a good training result always note the following:

- The more accurate you perform the labeling the better the result will be. You can start with a coarse labeling (as indicated in the image above) and then check the result for problematic areas where you should refine the labeling (as shown in Fig. 6.1)
- Accurate labeling is generally preferred over "just labeling everything" roughly.
- Take care to also label some areas which contain edges of objects and transitions between two classes.
- Really use an iterative approach: check the segmentation / training results before labeling huge amounts of pixels.

12.13.6.2 Importing Models

Prerequisite

✓ You have completed the general preparations.

✓ You have a trained model (*.czmodel file) or a pre-trained neural network available which you want to import.

✓ You are on the **Analysis** tab.

1. In the **Intellesis Trainable Segmentation** tool, click on **Options** and select **Import**.
2. In the file browser, select the model *.czmodel file or your network from the file system. The network can also be imported by selecting the respective JSON file.
3. Click on **Open**.

   → The model will now be available in the dropdown list.

4. Select the model and click on **Start Training** to work with the model, e.g. if you want to train more details.
   If you want to use the model for image processing switch to the **Processing** tab. In the **Trainable Segmentation** processing function you can select the imported model and apply it to the desired images/data sets.

See also

Using a Trained Model for Image Processing [▸ 434]
Using a Trained Model for Image Analysis [▸ 435]

12.13.6.3 Exporting a Model

Prerequisite ✓ You have created and selected a model for advanced image segmentation.

1. On the **Analysis** tab, in the **Intellesis Trainable Segmentation** tool, click on **Options** and select **Export**.
   If you want to export the full model containing all images select **Export with Images** (see also *Exporting with Images [▸ 430]*).
2. Select the file location and click on **Save**.

The model will be exported as ***.zip** file which contains the trained segmenting routine as well as the images which were used for training. In case of the option where the images are not included, only the model files itself will be exported. Such a model is meant to be used for segmentation purposes or to create an Image Analysis Setting, but not for the Training Process anymore.

12.13.6.4 Exporting with Images

You can export a training model as a zip file, so that you can continue to use them in other programs.

Prerequisite ✓ You have selected a trained model with images.

1. On the **Analysis** tab, in the **Intellesis Trainable Segmentation** tool, click on **Options** and select **Export With Images**.
   → The file explorer opens.
2. Navigate to the folder where you want to store the training model and click on **Save**.

You have exported your model with all images.

12.13.6.5 Renaming a Model

Prerequisite ✓ You have selected a training model.

1. On the **Analysis** tab, in the **Intellesis Trainable Segmentation** tool, click on **Options** and select **Rename**.
   → The **Model** field is editable.
2. Enter a new name for the model and save it.

12.13.6.6 Cloning a Model

Prerequisite ✓ You have selected a training model.

1. On the **Analysis** tab, in the **Intellesis Trainable Segmentation** tool, click on **Options** and select **Clone**.
   → The name of the currently loaded model disappears and the **Model** field is editable.
2. Enter a new name for the model and save it.

You have cloned an existing model.

12.13.6.7 Creating Analysis Setting

1. On the **Analysis** tab, in the **Intellesis Trainable Segmentation** tool, click on **Options** and select **Create Analysis Setting**.
   → The file browser opens with the default location for image analysis settings.
2. If you want to change the location, select a new path for saving the setting.
3. Click on **Save**.

You have now created and saved an image analysis setting.

For more information, see also *Using a Trained Model for Image Analysis [▸ 435]*.

12.13.6.8 Deleting a Model

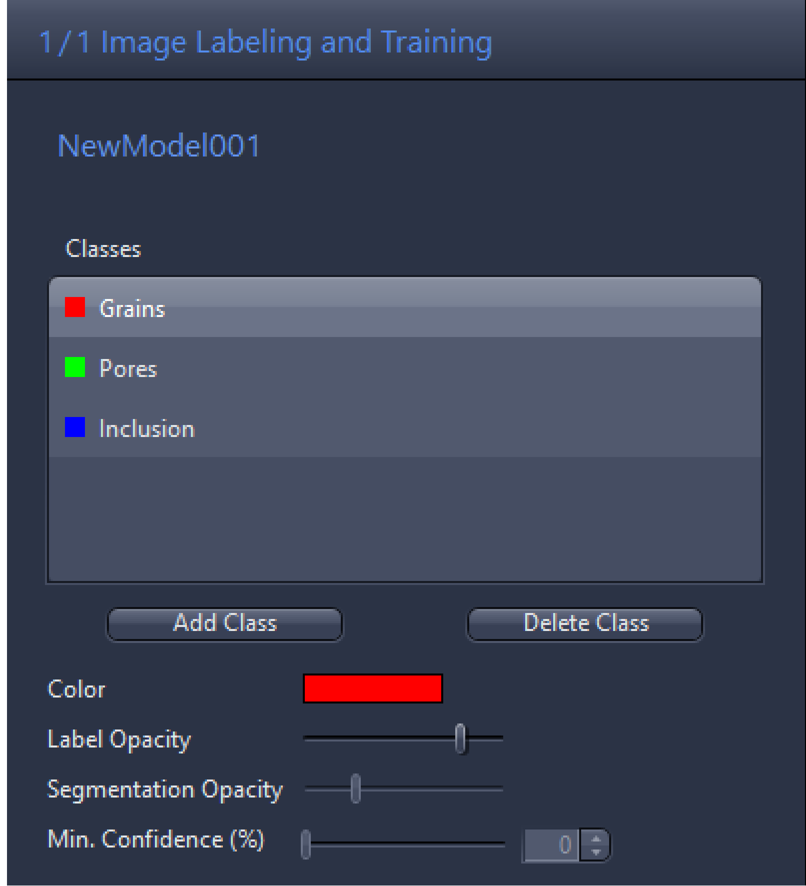
Prerequisite ✓ You have selected a training model.

1. On the **Analysis** tab, in the **Intellesis Trainable Segmentation** tool, click **Options** and select **Delete**.
   → The **Deleting Model** dialog opens.
2. Click on Yes to confirm that you want to delete the model.

You have deleted the model. The model you worked with before is selected.

12.13.7 Editing Classes

1. To add a new class to the **Classes** list, click on **Add Class**.
   → The classes have a random color by default.



2. To change the color of a class, select the class and click on the colored rectangle next to **Color**.
   → You see the **Color Selection** dialog.
3. Select a new color from the list.
4. To change the opacity of the labels within the image, adjust the **Opacity** slider.
5. To rename a class double click on the class entry and enter a new name. Press *Enter* or click on the **Save** icon to save the new name.
6. To delete the selected class, click on **Delete Class**.

12.13.8 Importing Labels from Binary Mask

With this class specific function you can import binary images from an external source as labels for the current selected class. This is helpful when the "ground truth" for a specific image is available or when you want to use an image obtained by a different modality.

Info

Be aware that this function overwrites existing labels for this class and that this functionality can possible create a huge number of labels that might lead to memory issues depending on the system configuration and the selected feature extractor.

Prerequisite
- ✓ The label image to be imported has exactly the same dimension in XY as the currently selected training image.
- ✓ You have opened the **Intellesis Trainable Segmentation** Wizard. For more information, see *Creating a New Model [▸ 425]*.

1. Right-click a class and select **Import Labels from Binary Mask**.
   → The Explorer opens.
2. Navigate to the label image you want to import, and click **Open**.

The imported image is displayed in the **Image** view. The displayed labels have the color of the selected class and fit exactly with the class of the loaded image.

12.13.9 Converting segmentations to labels

With this function you can convert the result of a segmentation directly to labels and thereby increase the number of labels for the next training step.

Prerequisite
- ✓ You have opened the**Training** user interface. For more information, see *Creating a New Model [▸ 425]*.
- ✓ You have performed a segmentation.

1. Right-click a class and select **Segmentation to Labels**.

The segmentations are converted to labels.

12.13.10 Using neural networks for Intellesis

In ZEN you can use pre-trained neural networks as models for image segmentation. You can use networks provided by Zeiss or load your own networks. Each network has to be imported to ZEN via the **Intellesis Trainable Segmentation** tool as a model. For more information, see *Importing Models [▸ 429]*.
After the import the network can be used as a normal segmentation model for the following workflows:

- Segment single channel images using the respective image processing function, see also *Using a Trained Model for Image Processing [▸ 434]*.
- Create an image analysis setting based on the network (no hierarchy), see also *Using a Trained Model for Image Analysis [▸ 435]*.
- segment a specific class in the *Automatic Segmentation [▸ 779]* step of the Image Analysis Wizard.

Using networks provided by Zeiss

Zeiss provides some pre-trained networks for you to use (subject to change without notice). These networks are available for download on the ZEISS GitHub page for Open Application Development (OAD) and can be found inside the Machine-Learning section under Model Downloads.

Note: These networks are copyright protected!

Condition of Use
These pre-trained networks were trained with "best-effort" on the available training data and is provided "as is" without warranty of any kind. The licensor assumes no responsibility for the functionality and fault-free condition of the pre-trained network under conditions which are not in the described scope. Be aware that no pre-trained network will perform equally good on any sample, especially not on samples it was not trained for. Therefore, use such pre-trained networks at your

own risk and it is up to the user to evaluate and decide if the obtained segmentation results are valid for the images currently segmented using such a network. By downloading you agree to the above terms.

Detailed Information about pre-trained DNNs

Such networks are specific for the application they have been trained for. Detailed information can be provided on demand.

Using your own networks

You can also train and use your own networks. To be able to use your own networks in ZEN, your networks have to fulfill certain specifications detailed in the ANN Model Specification.
An example of how to train a model and convert it into a czmodel can be found here: Importing External Networks in ZEN. It also explains the usage of the PyPi package which is free to use for everybody.

12.13.11 Using a Trained Model for Image Processing

Prerequisite

- ✓ You have a trained model available for automatic image segmentation.
- ✓ You have opened the image which you want to segment under **Images & Documents**.

1. On the **Processing** tab under **Method** open the group **Intellesis Trainable Segmentation** and select the **Segmentation** entry.
2. Open the **Method Parameters** and select the trained model from the **Model** list. Note that the model must be trained on images with similar features otherwise the segmentation will not work properly.
3. Select the desired **Output Format**.
   If you select **Multi-Channel**, the result will be a multi-channel image, where every class that was defined in the trained model will be in their own channel. This output format can be easily viewed inside the ZEN **3D view** and can be combined with the original image data easily.
   If you select **Labels**, you will get an image with one channel, where the pixels belonging to the different classes will be labeled with different colors and will be represented by distinct pixel values.
4. Under **Input Parameters** select the image which you want to segment. Note that it must be already opened in the ZEN software, otherwise it will not be available in the list.
5. Click on **Apply**.
   - → The automatic image segmentation using the trained model is performed.
   - → After a short while you will get two resulting images, depending on the output format:
     - the multi-channel or labels image and
     - the confidence map.

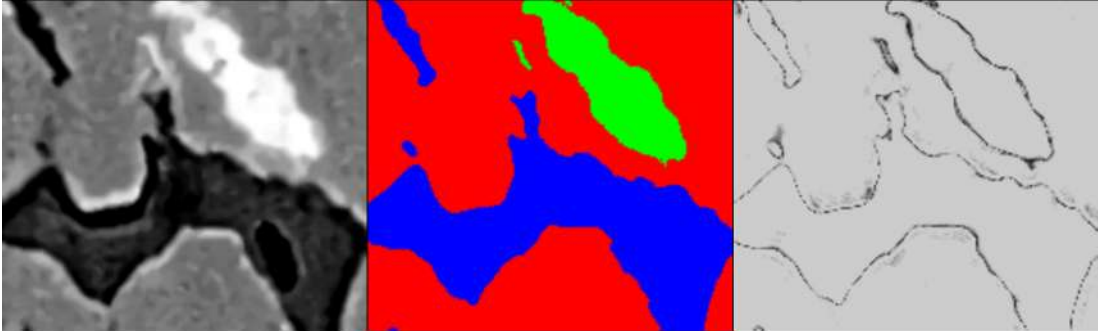


Fig. 27: The image shows (from left to right): original image, segmented image, confidence map

See also

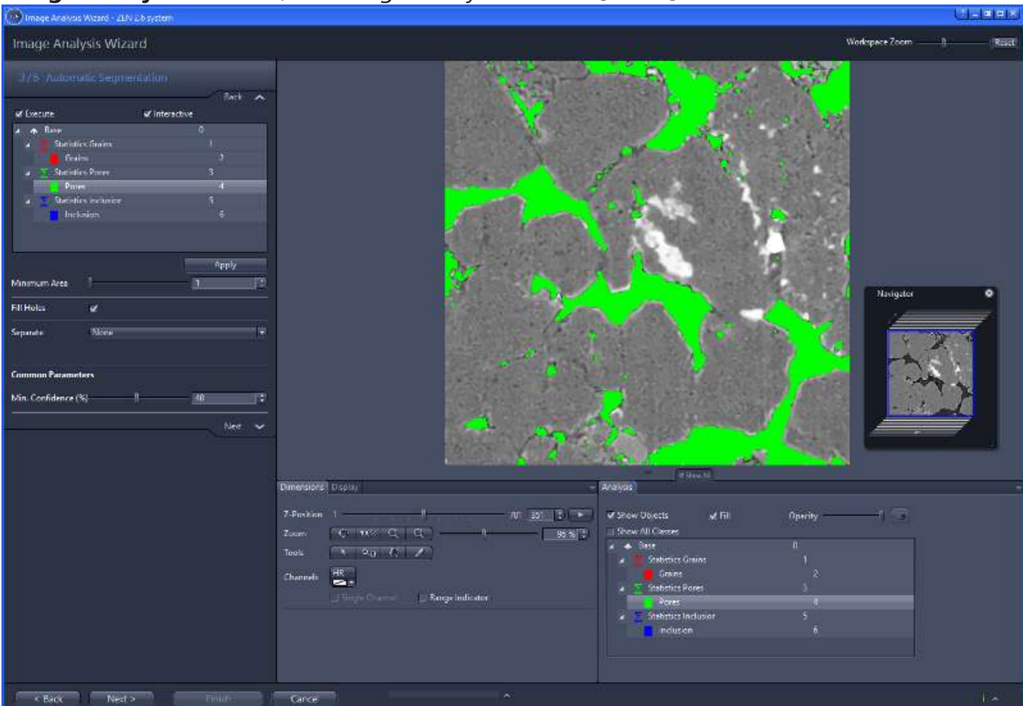
Segmentation [▸ 441]

12.13.12 Using a Trained Model for Image Analysis

Once having trained a model for segmentation you can use it also in the **Image Analysis** wizard of the software for further analysis. In order to use the trained model, you must first create a new image analysis (IA) setting (*.**CZIAS** format) first.

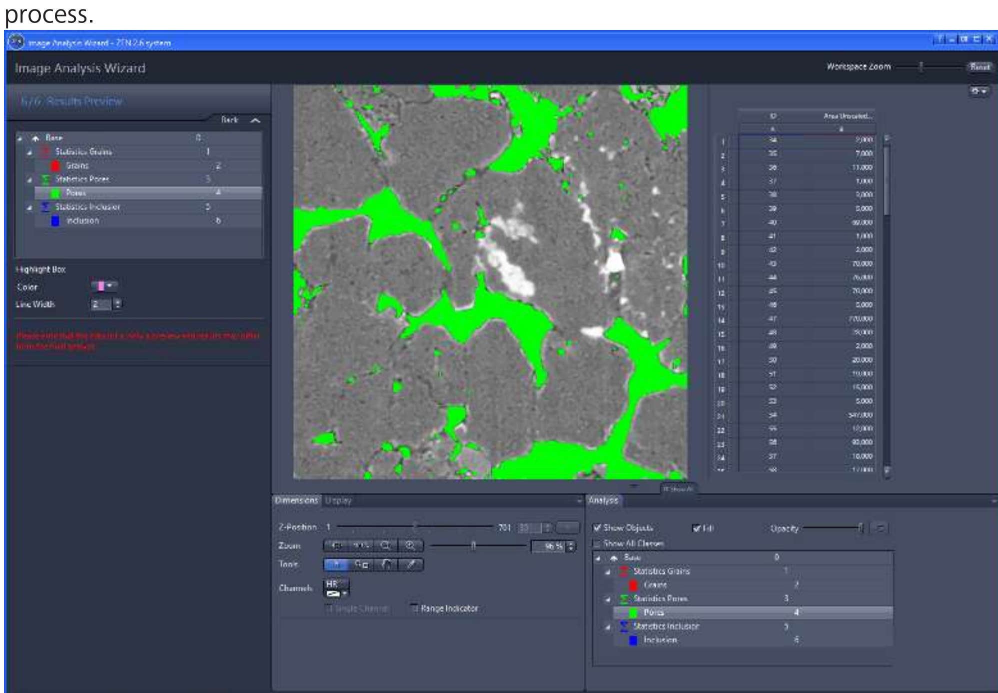
Prerequisite ✓ You are in the **Trainable Segmentation** tool.

1. Select the trained model of which you want to create an analysis setting.
2. Click on the **Options** and select **Create Analysis Setting**.
   → The dialog for saving the setting opens. The setting will be saved as *.czias file in the ZEN default folder for image analysis settings (usually under **User/Documents/Carl Zeiss/ZEN/Documents/Image Analysis Settings**).
3. Click on **Save**.
4. Now change to the **Image Analysis** tool and select the setting from the dropdown-list. Note that the setting will be only available in the dropdown list when you have used the default folder for saving. Otherwise the setting must be loaded from the file system (specific location) via the **Import** option (see *Importing Models [▸ 429]*).
   → The model will be loaded with its predefined classes.
5. You can now continue with setting up an image analysis. For more information about the **Image Analysis Wizard**, see *Image Analysis Wizard [▸ 777]*.



   → Sandstone Dataset segmented using **Intellesis** inside the **Image Analysis Wizard** showing the actual segmentation step. Instead of conventional thresholds, the classifier will be used to identify pixels.
   → It is possible to allow only for pixel above a certain classification confidence (valid for all classes) using the **Min. Confidence (%)** parameters.

→ The binary functions Fill Holes and Separate will be only applied on the resulting binary masks from the classification and are therefore independent from the actual classification process.



→ Sandstone Dataset segmented using **Intellesis** inside the **Image Analysis Wizard** showing the measurement results for one particular class (shown in green).

→ You can also use the IP function inside the **ZEN Blue Batch** Tool similar to all the other functions to segment several images using different models at once in one run.

12.13.13 Using Intellesis within OAD

The **Intellesis Trainable Segmentation** module allows to use the **Trainable Segmentation** processing function within the ZEN **Open Application Development (OAD)** environment.

Method / Command	Description
Zen.Processing.Segmentation.**TrainableSegmentation** (Input, Model, Output Format)	Function to segment an image using a trained model. The output result is an image.
▪ Input	ZenImage - Defines the input image to be segmented.
▪ Model	ModelName - Defines the name of the model.
▪ Output Format: SegmentationFormat.**MultiChannel** SegmentationFormat.**Labels**	Function to segment an image using a trained model. The output result is an array of images containing the segmented image and the confidence map.
Zen.Processing.Segmentation.**TrainableSegmentationWithConfidenceMap**	Addresses the Trainable Segmentation function including a confidence map.
▪ Input	ZenImage - Defines the input image to be segmented.

Method / Command	Description
▪ Model	ModelName - Defines the name of the model.
▪ Output Format: SegmentationFormat.**MultiChannel** SegmentationFormat.**Labels**	SegmentationFormat - Optional argument; Defines the desired output format, e.g. Multi-Channel or Labels
Zen.Processing.Segmentation.**MinimumConfidence**	Addresses the Minimum Confidence function.
▪ Input	ZenImage - Defines the input image to be segmented.
▪ Model	ZenImage - Confidence map containing the confidence values in %.
▪ Threshold	Minimum Threshold – value in % - only pixel inside mask >= this values will be kept.
ZenIntellesis.**GetAvailableFeatureSets**()	Returns all available feature sets as an array of strings.
ZenIntellesis.**GetAvailablePostProcessings**()	Returns all available post-processing options as an array of strings.
ZenIntellesis.**ImportModel**(modelfile, allowOverwrite)	Imports a model file into the model repository and overwrites an existing one, if the option was set to True. Returns a ZenIntellesisModel.
▪ modelfile	File path to modelfile to be imported.
▪ allowOverwrite	Allows overwriting an existing model.
ZenIntellesis.**ListAvailableSegmentationModels**()	Lists all available segmentation models. Returns an array of ZenIntellesisModels.

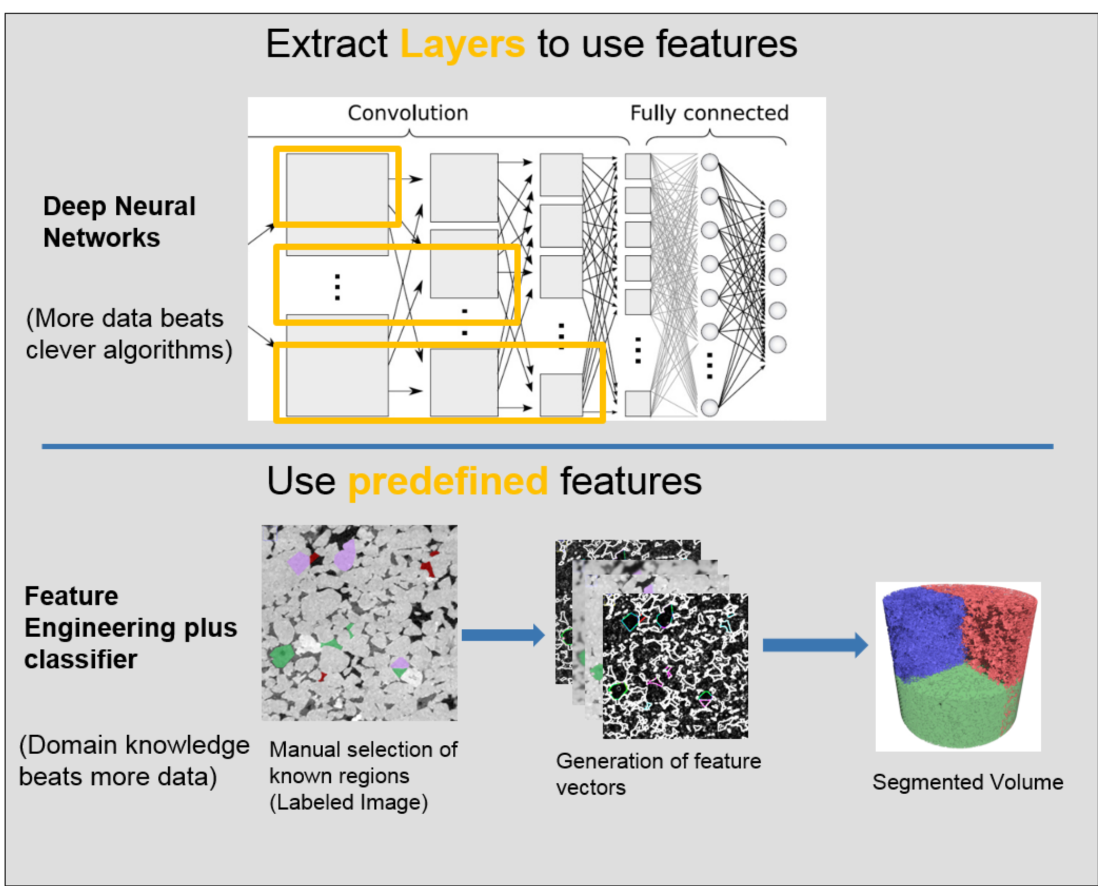
12.13.14 Remarks and Additional Information

- Segmentation Performance in general depends among other factors on the system performance, the **available and free RAM and GPU memory**.
- **Whenever using ZEN Intellesis Trainable Segmentation it is strongly recommend not to use other memory- or GPU-intensive applications at the same time**.
- **The training of Intellesis models is CPU / GPU specific.** A model trained on GPU only runs on a GPU machine. If a model trained on GPU is transferred to a CPU-only machine, the model has to be retrained to run on this machine. The reason is that Tensorflow will give slightly different results depending on whether the Tensorflow-CPU or Tensorflow-GPU is used.
- Deep Feature Extraction will use the GPU (NVIDIA only) if present on the system. It is recommended to use an GPU with at least 8GB of RAM.
- The installation of the GPU version of **Intellesis** can be selected during the installation process of ZEN itself.
- When installing the GPU libraries it is required to use the latest drivers which can be obtained from the NVIDIA homepage (https://www.nvidia.com/Download/index.aspx?lang=en-us).

- In case of using an approved ZEISS workstation the latest drivers can be found on the installation DVD.
- When using Deep Feature Extractor on a GPU system, Tensorflow will occupy only as much as GPU RAM as needed to ensure system stability. When the segmentation is finished this GPU memory released automatically (with the current version).
- Therefore, when starting another GPU-intensive application, for example GPU-DCV, the GPU memory cannot be used by this new process and a CPU fallback will be used or performance issues may occur.
- In such a case, restart ZEN to free all possible GPU memory and then start using GPU-DCV (or similar applications).

12.13.15 Functions & Reference

12.13.15.1 Feature Extractors



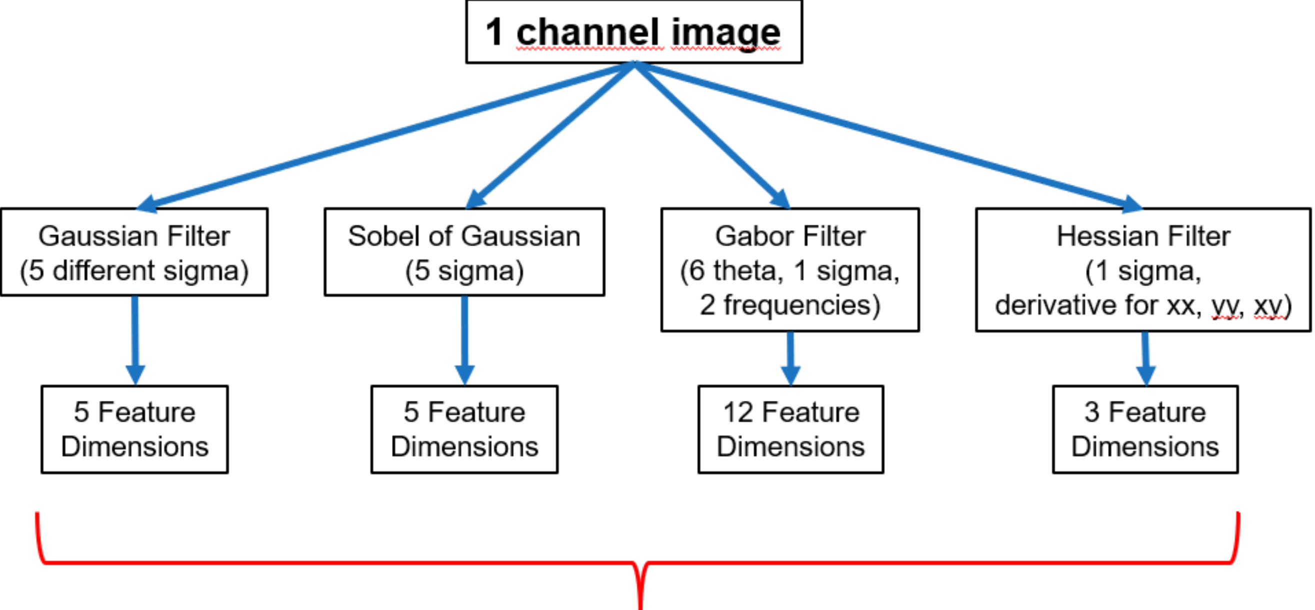
12.13.15.1.1 Intellesis Basic Features

- For calculating the features various filters with various filter sizes and parameters are applied to the region around this pixel (2D Kernels).
- Results are concatenated and yield the final feature vector describing the pixel.

12.13.15.1.1.1 Basic Features 25

Used Filters:

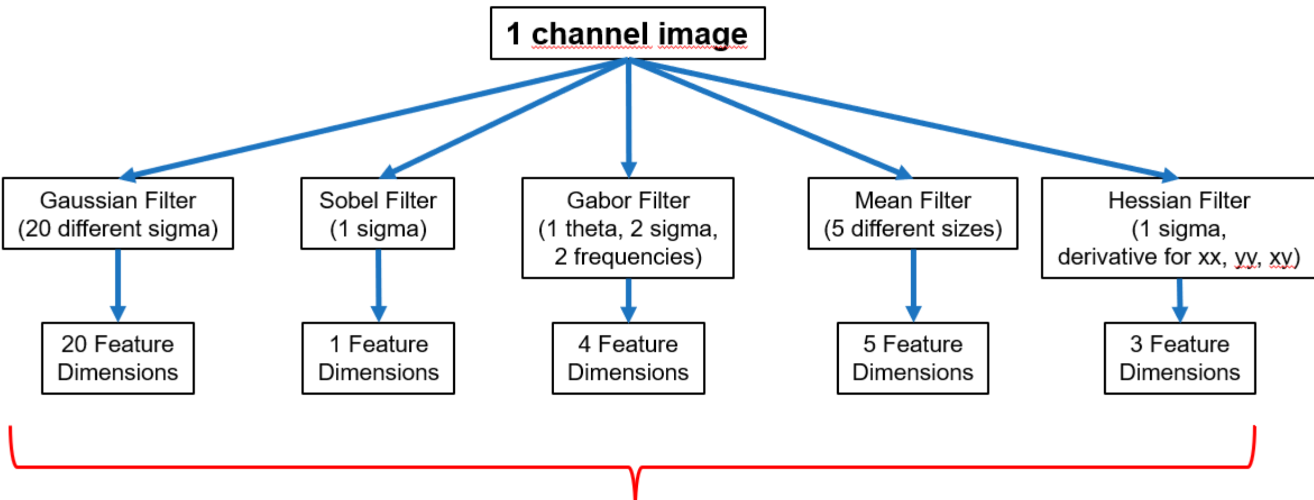
- Gaussian filter (5 different sigma) = 5 feature dimensions
- Sobel filter (5 sigma) = 5 feature dimension
- Gabor filter (6 theta, 1 different sigma, 2 different frequencies) = 12 feature dimensions
- Hessian filter (1 sigma) = 3 feature dimensions (one for derivative in direction xx, one for derivative in direction xy and one for derivative in direction yy)



12.13.15.1.1.2 Basic Features 33

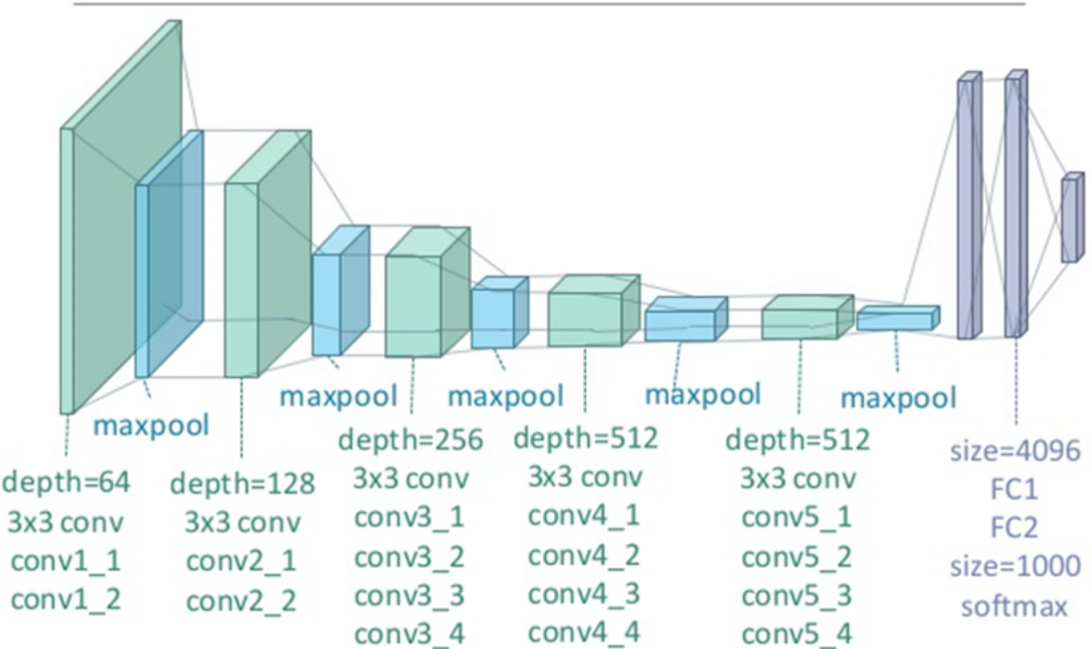
Used Filters:

- Gaussian filter (20 different sigma) = 20 feature dimensions
- Sobel filter (1 sigma) = 1 feature dimension
- Gabor filter (1 theta, 2 different sigma, 2 different frequencies) = 4 feature dimensions
- Mean filter (5 different sizes) = 5 feature dimensions
- Hessian filter (1 sigma) = 3 feature dimensions (one for derivative in direction xx, one for derivative in direction xy and one for derivative in direction yy)



12.13.15.1.2 Intellesis Deep Features

- Entire image as input for pre-trained network.
- **Note:** If you use the CPU for segmentation with Deep Feature sets, the results can be different on different machines because they are hardware (CPU) dependent.
- Take the output from an intermediate layer of that network as feature vector, e.g. output from layer 3 was processed by preceding layers 1 and 2.
  - Deep Features 50: Using layer 2 with reduced feature dimension = 50
  - Deep Features 64: Using layer 1 with full feature dimension = 64
  - Deep Features 70: Using layer 3 with reduced feature dimension = 70
  - Deep Features 128: Using layer 2 with full feature dimension = 128
  - Deep Features 256: Using layer 3 with full feature dimension = 256



12.13.15.2 Image Processing Functions

12.13.15.2.1 Minimium Confidence

Parameter	Description
Threshold slider	Adjusts the minimum confidence level in %.

12.13.15.2.2 Segmentation

Using the **Segmentation** image processing function you can apply a trained segmentation model to an image/data set.

Parameter	Description
Model	Select the trained model here.

Parameter	Description
Output Format	When applying the **Segmentation** processing function to an image you will always get two output images. The processed image and the confidence map. The following output formats for the processed image are available.
- Multi-Channel	If selected, the output image will be a multi-channel image. Each class which is defined within the model will result in a separate channel.
- Labels	If selected, the output image will be a single-channel image.

See also

Using a Trained Model for Image Processing [434]

12.13.15.2.3 Utilities

Here you can convert an output image generated with the **Segmentation** IP function according to your needs:

Parameter	Description
Labels to Channels	Converts the resulting image with the output format "**Labels**" to a multi-channel image.
Channels to Labels	Converts the resulting image with the output format "**Multi-Channel**" to an image containing a single channel image with labels. Under **Parameters** you can additionally adjust the Unlabeled Pixel Value and the Output Pixel Type (8 Bit B/W or 16 Bit B/W).

12.13.15.3 Intellesis Trainable Segmentation Tool

Parameter	Description
Model	Shows the selected model. If you have several models available you can select the corresponding model from the drop-down list.
Options	
– New	Creates a new, empty model. For more information, see *Creating a New Model [425]*.
– Rename	Renames an existing model. For more information, see *Renaming a Model [430]*.
– Delete	Deletes an existing model. For more information, see *Deleting a Model [431]*.
– Create Analysis Setting	Creates and stores a *.czias file in the specific folder for image analysis settings. The file can then be used in the **Analysis Wizard**. For more information, see *Creating Analysis Setting [431]*.
– Clone	Clones (duplicates) an existing model. For more information, see *Cloning a Model [431]*.

Parameter	Description
– Export	Exports the model to the file system. For more information, see *Exporting a Model [▸ 430]*.
– Export With Images	Exports the model including all images to the file system. For more information, see *Exporting with Images [▸ 430]*.
– Import	Imports a model to the ZEN software. For more information, see *Importing Models [▸ 429]*.
Description	Displays a descriptions of the selected model.
Postprocessing	Selects a postprocessing. For more detailed information, see *Postprocessing Options [▸ 423]*
– No Postprocessing	No postprocessing will be applied on the images.
– Conditional Random Field	If selected, this post processing function is applied to the output of the pixel classification. The CRF algorithm tries to create smoother and shaper borders between objects by re-classifying pixels based on confidence levels in their neighborhood. Note: If CRF is activated, the returned confidence map does not reflect the outcome of the majority votes of all decision trees of a specific class anymore. Therefore, a map containing only ones will be returned when the CRF postprocessing option is activated.
Start Training	Opens the Training UI, see *User Interface - Training [▸ 421]*

12.14 Macro Environment

The acronym OAD for Open Application Development is a term describing both the OAD platform on ZEN as well as the process of developing applications on it. The platform has been made available for our customers to enhance the functionality of ZEN in a flexible way. With OAD typical microscopy workflows can be integrated into the ZEN software. A short list of OAD highlights: Macro Interface to access the major functionality of ZEN and its objects and the access to external libraries like the .Net Framework to significantly enlarge the field of application.

The software offers the following components which we regard as main parts for Open Application Development (OAD):

- Macro Runtime Environment (integrated)
- Macro Recorder
- Macro Editor
- Macro Debugger
- Macro Interface (Object Library)
- ImageJ Extension

Basic functionality

All ZEN Products (ZEN lite excluded) come with a basic macro functionality which allows to play existing macros within the software (**Macro tool**).

Info

Within ZEN you can only run **.czmac** macro files which are aquired or saved in the ZEN macro environment. To run your macros they must be located in the folder: …/**User**/**Documents**/**Carl Zeiss**/**ZEN**/**Documents**/**Macros**.

Advanced functionality

The Macro Recorder, Editor and Debugger form the Integrated Development Environment (IDE) comes with the ZEN module **Macro Environment**. The IDE for the Macro Environment consists of two parts. There is a reduced IDE in the **Right Tool Area** which you find within the *Macro tool [▸ 804]*. The full blown IDE is available in the Macro Editor dialog and allows users to generate and work with macros similar to Excel/Word macros. The Macro Interface is built into the software and therefore not a separate product. The **ImageJ** Extension is the first extension for ZEN and will be free of charge. It will not be available in ZEN lite.

User forum

A user forum was established to allow users to exchange macros and to discuss solutions. You will find a lot of example macros and further documentation there. The user forum can be reached under www.zeiss.com/ZEN-OAD.

12.14.1 Running an existing macro

Prerequisite

- ✓ You work with a licensed version of the software, e.g. **ZEN pro**, **desk** or **system**. Note that the macro environment for ZEN lite is not available.
- ✓ You have not licensed the **Macro Environment** module.
- ✓ You have a macro file available that you want to play in ZEN.

1. Copy your macro file in the following folder:
   .../User/My Documents/Carl Zeiss/ZEN/Documents/Macros.
2. Start the software.
3. In the **Right Tool Area** open the **Macro** tool.
   → You see your macro in the list under **User Documents**.
4. Select your macro.
5. Click on the **Run** button.

Your macro is executed. You have successfully played a macro in ZEN.

12.14.2 Recording a macro

This guide shows how to record a macro of a simple processing workflow.

Prerequisite

- ✓ You have licensed the **Macro Environment** module.
- ✓ You are in the **Right Tool Area** in the **Macro** tool**.**

1. Click on the **Record** button.
2. Load a color image via the menu **File > Open**…
3. Go to the **Processing** tab.
4. Under **Method** select **Edges > Sobel**.
5. Under **Method Parameters > Normalization** select the entry **Clip.**
6. Under **Image Parameters** set your color image as **Input** Image.
7. At the top of the **Processing** tab, click on the **Apply** button.
   → The **Sobel** method will be applied to your image. The output image will be generated and opened in a new image container.
8. In the **Macro** tool click on the **Stop** button.

You have successfully recorded a macro for a simple processing workflow. The workflow can now be repeated automatically just by playing the recorded macro file.

12.15 Panorama

This module enables you to create overview images of large areas of your sample.

Prerequisites

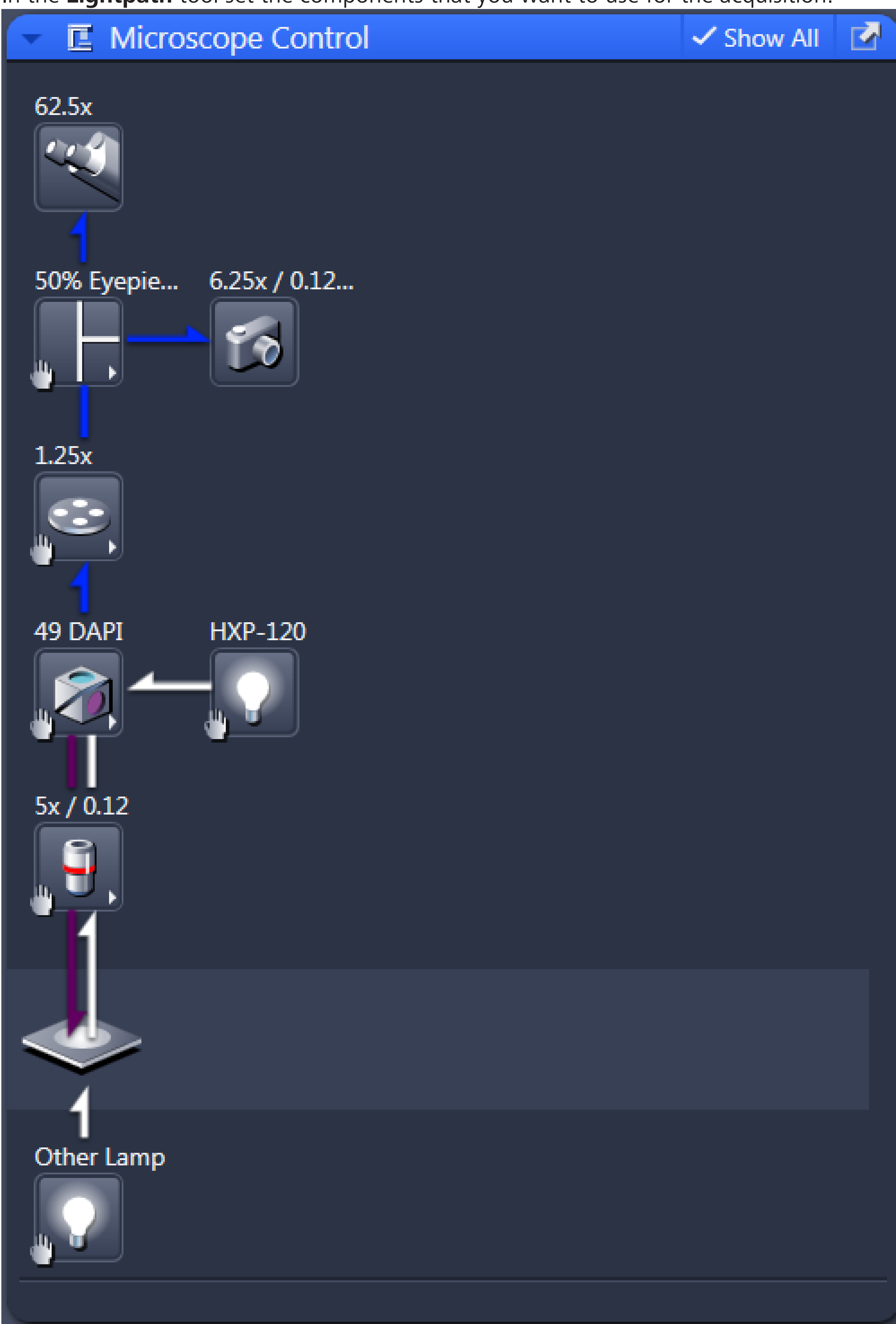
For the interactive panorama acquisition following prerequisites are necessary:

- All available microscope components in the **MTB** (MicroToolBox) have to defined correctly.
- The **Panorama** module is activated in the menu **Tools > Modules Manager**.

12.15.1 Prerequisites

Prerequisite ✓ You are on the **Locate** tab.

1. In the **Lightpath** tool set the components that you want to use for the acquisition.



→ All these settings will be stored as metadata of the image.

→ Additionally the optical components will be used to automatically determine the pixel size for the scaling.

2. Click on the **Live** button.

→ Now you see the cameras live image of your sample in the **Center Screen Area**.

3. Click on the **Set Exposure** button.

→ The exposure time will be calculated automatically.

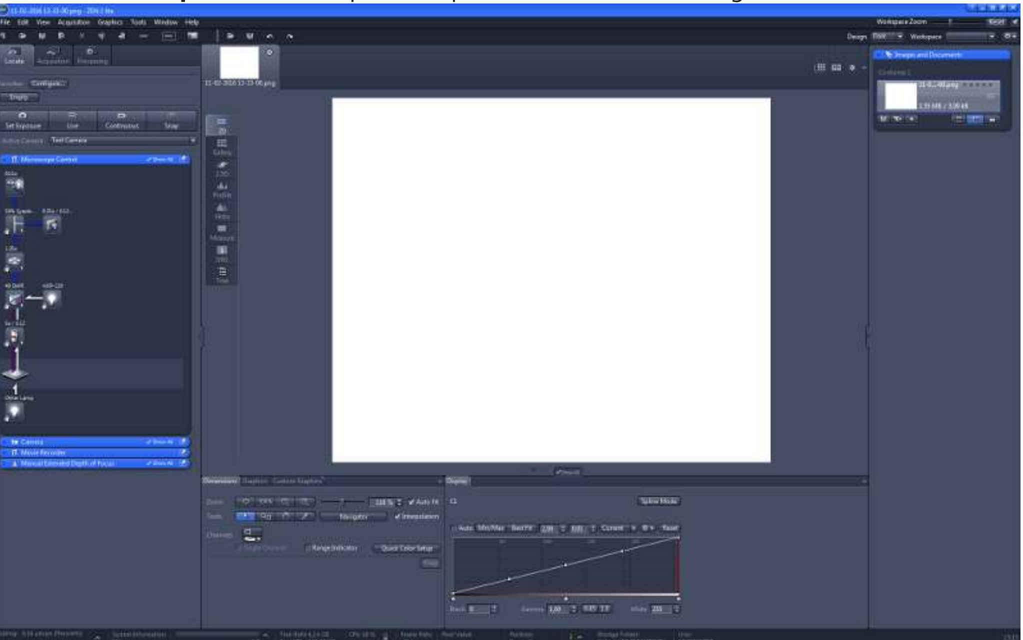
4. Alternatively you can set the camera parameters manually in the **Camera** tool.
5. Focus on your sample now.

You have completed the prerequisites for a panorama experiment.

12.15.2 Acquiring a Reference Image

Before starting the experiment itself, we recommend to acquire a reference image for the shading correction first. This image will be used later for processing the panorama image.

1. Move the sample to an empty field.
2. Click on the **Snap** button to acquire this position as reference image.



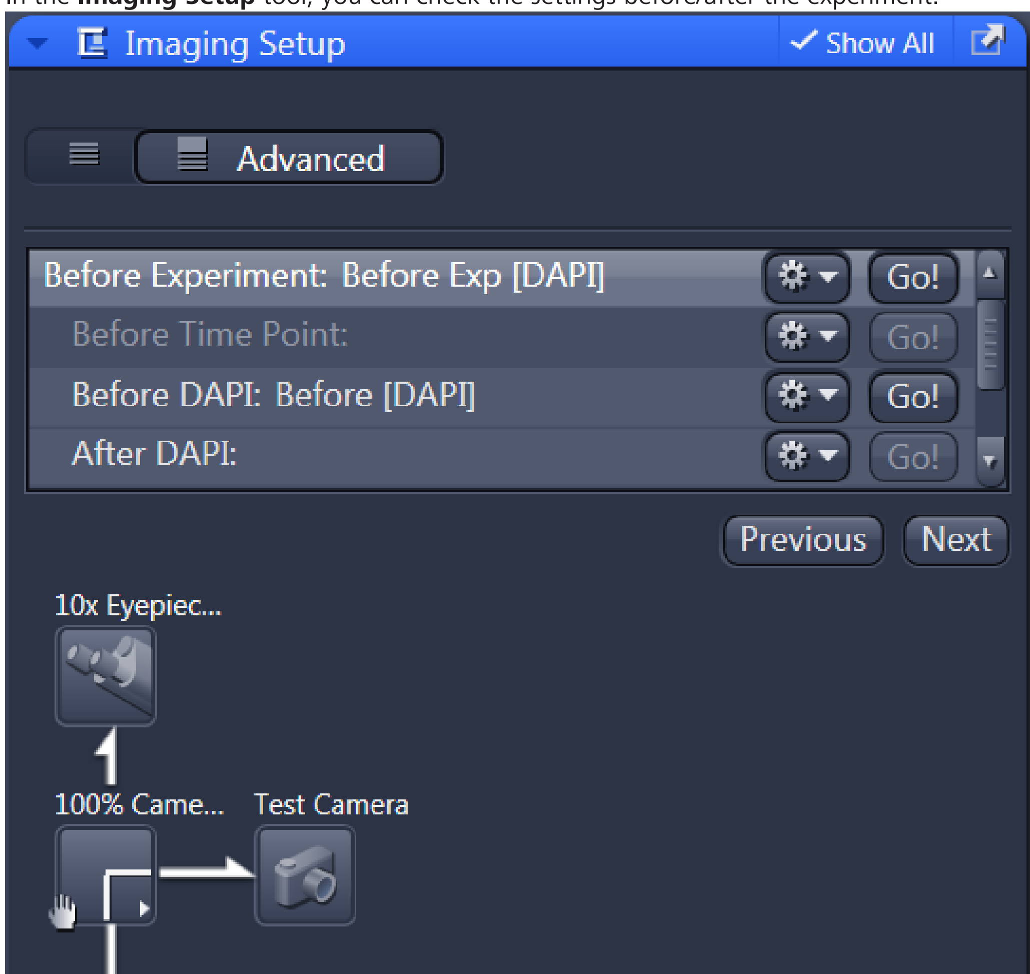
3. Save the image and let it opened in the background.

You have successfully acquired a reference image for the shading correction.

12.15.3 Settings for the Panorama Experiment

Prerequisite
- ✓ You are on the **Acquisition** tab.
- ✓ You have started the live mode via the **Live** button.

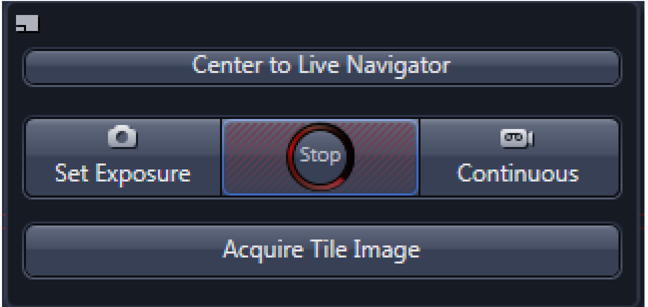
1. Activate the **Panorama** checkbox.
2. Move the sample to the desired start field of the panorama image to be acquired.
3. Check the defined exposure time again if necessary.
4. In the **Imaging Setup** tool, you can check the settings before/after the experiment.



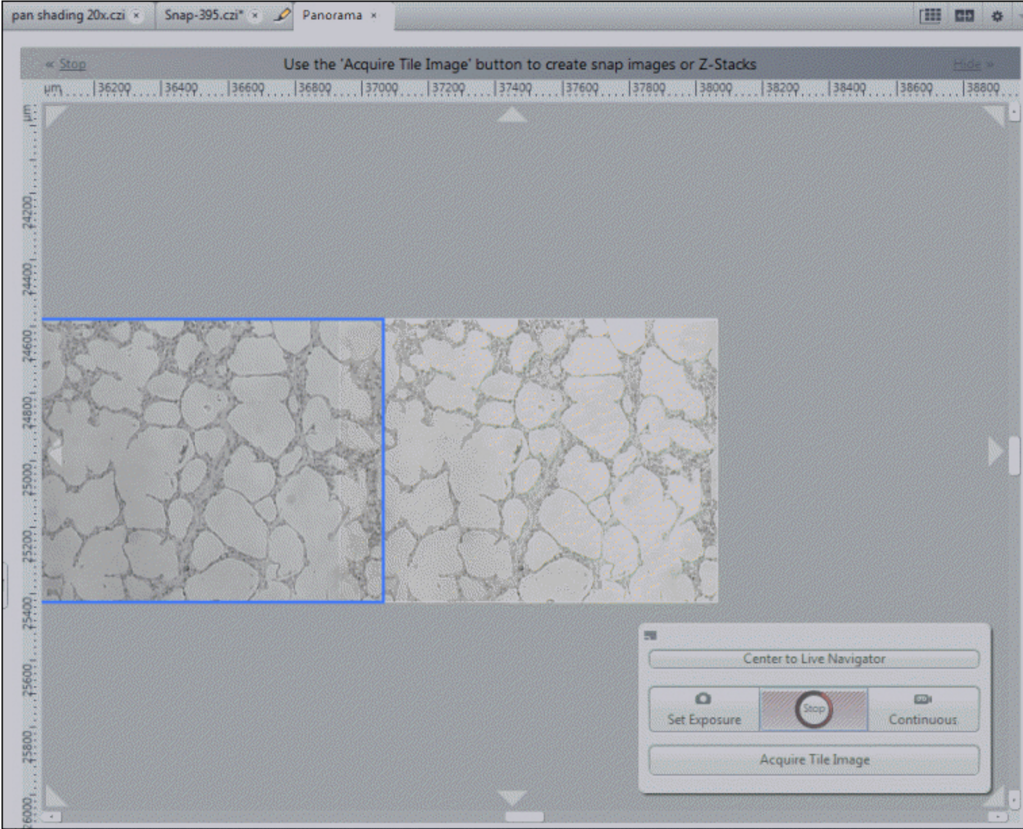
5. In the **Acquisition Mode** tool, click the **Get** button to transfer the active camera settings into the experiment.
   As an alternative you can define your experiment settings here as well.
6. In the **Panorama** tool you can adjust several options for automatic or manual stitching, if desired.
7. Finally save your experiment with a suitable name in the **Experiment Manager**.

12.15.4 Acquiring the Panorama Image

1. Click on the **Start Experiment** button to start the Panorama acquisition.
   → You will see the reduced display of the start image in the **Center Screen Area**.

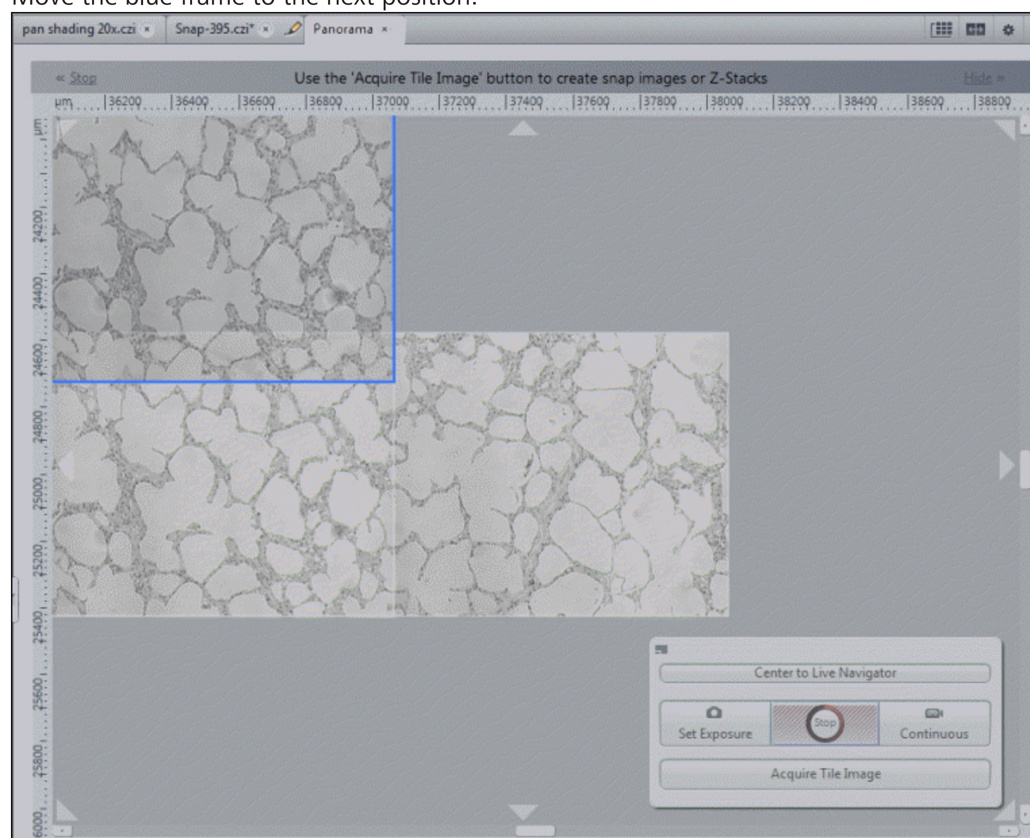


   → The displayed image is a live image. You can still change position and focus.
2. Click on the **Acquire Tile Image** button in the **Center Screen Area** to acquire the first tile image.
   → The image will be acquired and stored. The live image is still active as an overlay to the stored image.
3. Move the blue frame with the active live image in the desired direction aside the stored image by using the mouse.

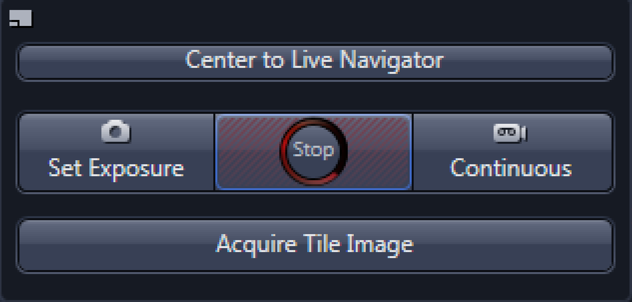


4. Now move the sample to the corresponding neighbor position using the microscope stage.
   → Try to position the structures in the overlap region as good as possible.
5. Click again on the **Acquire Tile Image** button to acquire this tile to the image.

6. Move the blue frame to the next position.



7. Continue these steps until you acquired the desired panorama image of your sample.
8. After acquiring the last tile image click on the **Stop** button to close the live mode.



9. Finally end the experiment via the **Stop** button on the **Acquisition tab**.
   → As a result you now see the recorded panorama image in a new image container.

10. Save the image as a raw image (*.**czi**).

You have successfully acquired and stored a panorama image.

Info

- You can adapt the size of image and surrounding area with the zoom keys *F7* and *F8* to your needs.
- Keep a sufficient overlap area of the live with the stored image.
- In the case of errors during the following processing steps you therefore always have access to the original image data.

12.15.5 Processing the Panorama Image

The next chapters will show you how to process panorama images with the **Stitching** processing function. Using this method you can correct an offset between the tile images. We will show you the different settings and make a comparison of the output images. So you can see which settings will give you the best result.

Prerequisites

Prerequisite ✓ You are on the **Processing** tab.

1. Open the **Method** tool and select in the group **Geometric** the **Stitching** function.

The following instructions will all base on this selection and show the different settings and results of this function.

12.15.5.1 Stitching (Defaults)

1. In the **Method tool** open the **Geometric** group**.**
2. Select the **Stitching** function.
3. Click on the **Apply** button in the **Processing** tab to start the processing.
   → The stitching process will take a while depending on the image size.

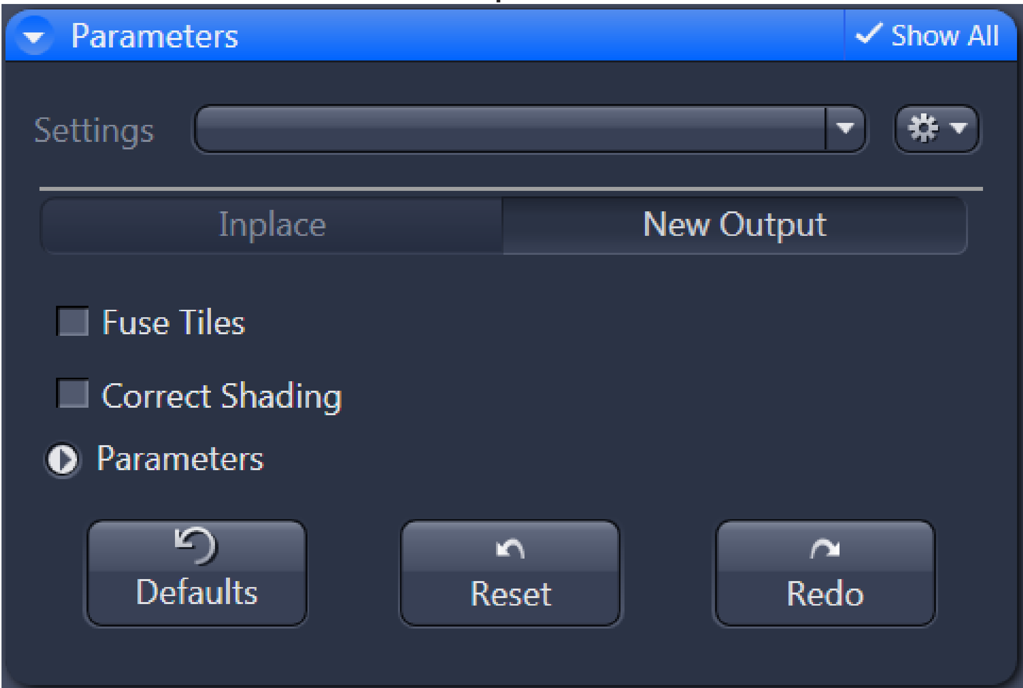
   → In the **Status bar** you can see the work in progress.

You have successfully used the **Stitching function**. As you still can see shadows and edges in the output image we will show you how to use the function receiving better results in the following chapters.

12.15.5.2 Stitching with Shading Correction

If your tile images contain a certain background shading you can correct this if you have acquired a reference image for the shading correction. This image has to be opened in the **Center Screen Area**.

1. Select in the tile image for the stitching in the **Input** tool as first input.
2. In the **Parameters** tool select the **New Output** button.



→ This will keep the original image and create a new output image.

3. Activate the **Correct Shading** checkbox.
4. Select the **Reference** entry from the dropdown list.

   → This will let you select your reference image which is opened in Center Screen Area.´
5. Now as a second input image select the reference image for the shading correction in the **Input** tool.
6. Click on the **Apply** button to start the processing.

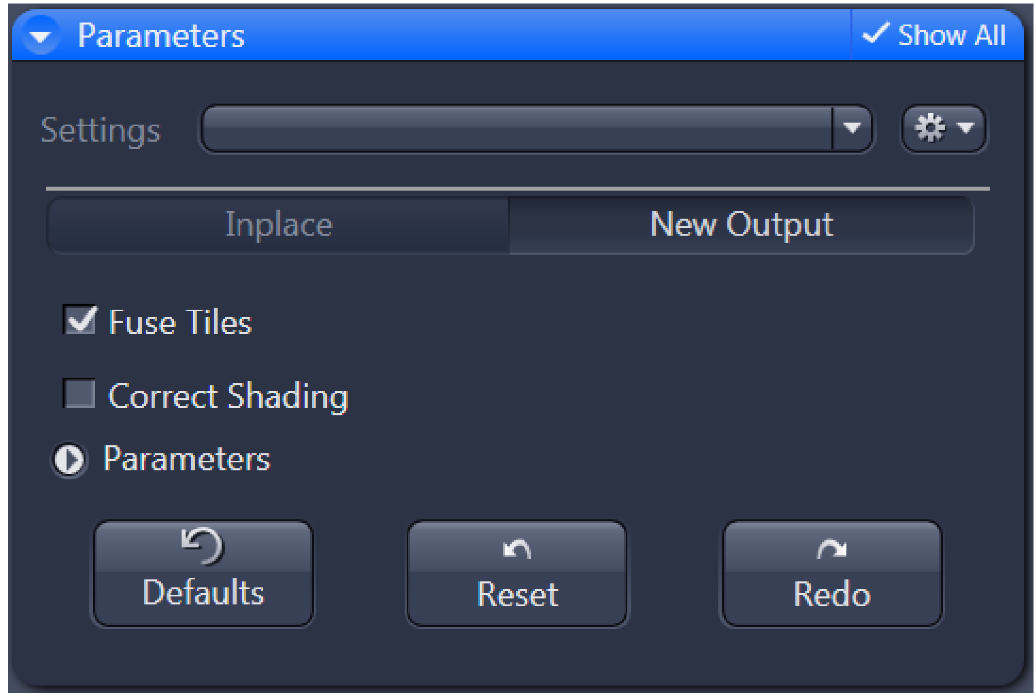
As a result you will get a stitched panorama image without any shading influences. The next chapter will show you how to get rid of the edges which are still visible between the tiles.

12.15.5.3 Stitching with Fuse Tiles

With very low shading content in the image you have an alternative method to homogenize the image transitions between the single tiles.

1. Under **Image Parameters** in the **Input** tool select the tile image for the stitching.
2. In the **Parameters** tool, click on the **New Output** button.

3. Activate the **Fuse Tiles** checkbox.



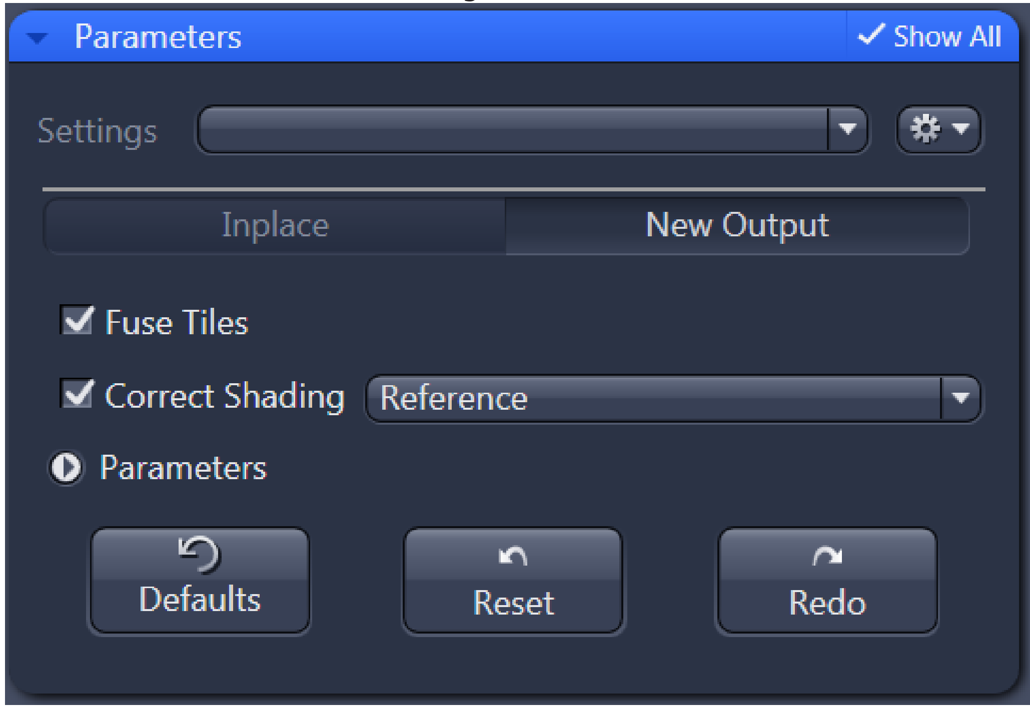
4. Click on the **Apply** button to start the processing.

This function will smoothly average all edge regions of the tiles via their grey values in order to avoid sharp edges. With high background shading this function alone will not be sufficient.

12.15.5.4 Stitching with Fuse Tiles and Shading Correction

For extreme cases you have the possibility to combine both transition corrections.

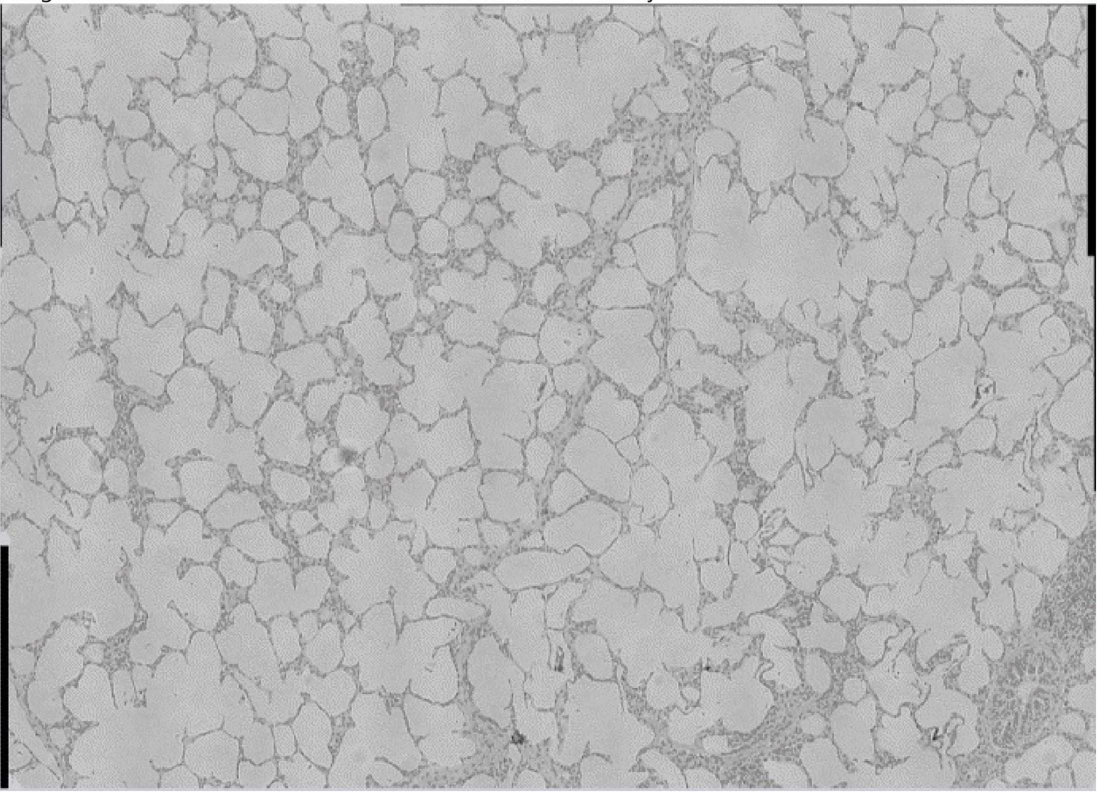
1. In the **Parameters** tool click on the **New Output** button.
2. Activate the checkboxes **Correct Shading** and **Fuse Tiles.**



3. Under **Image Parameters** in the **Input** tool select the tile image for the stitching and the reference image for the shading correction.

4. Click on the **Apply** button.

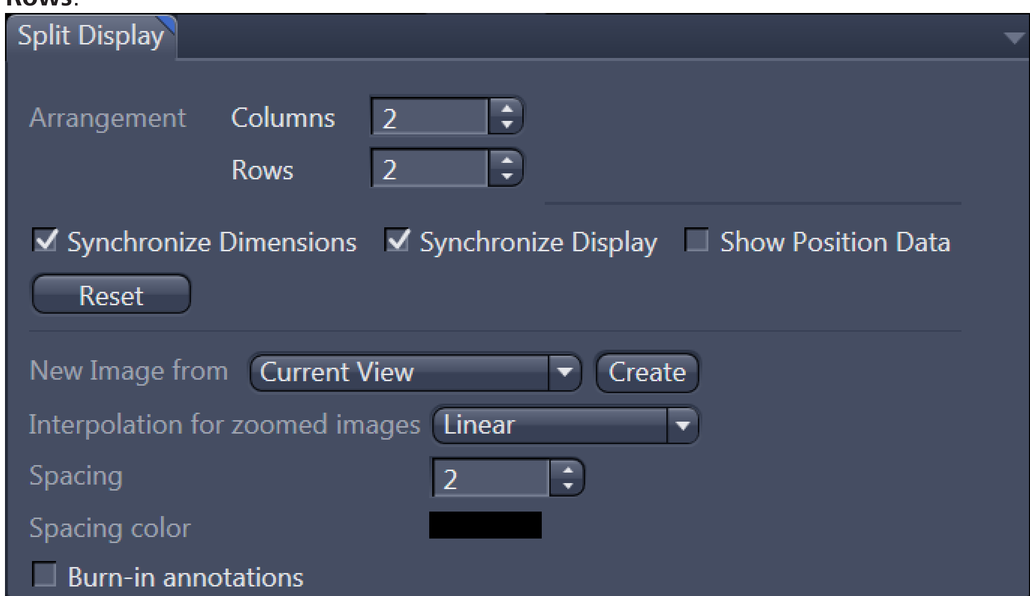
Both settings will be applied to the image. With this method you will receive a perfectly stitched image with no visible transition areas between the tiles any more.



12.15.5.5 Image Comparison via Split Display

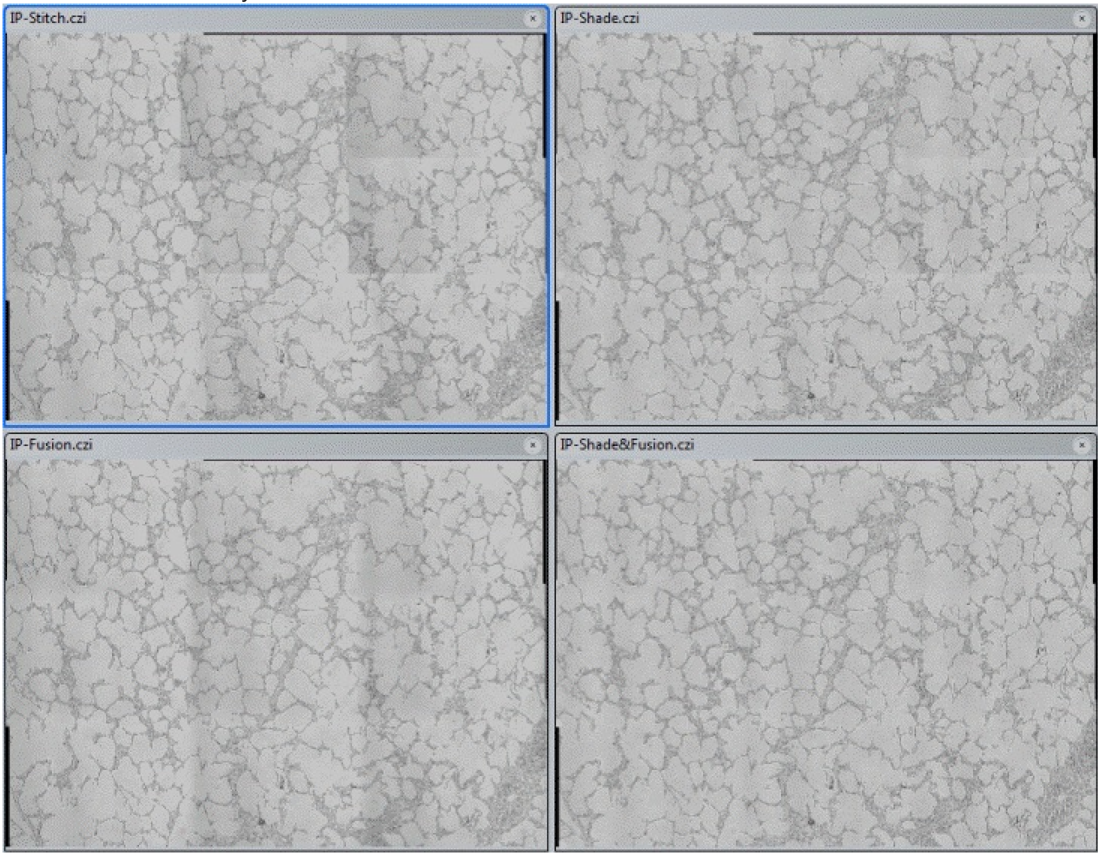
You can create a Multi Image to compare the different results of your processed images via the **Splitter-Mode.**

1. To compare different images, you can select the **Split Display** via the **Create New Multi Image** button.
2. On the **Split Display** tab you can define how many images shall be displayed in X- and Y-direction aside each other and how they shall be synchronized, e.g. 2 **Columns** and 2 **Rows**.



3. Move each of the different panorama images via drag&drop from the **Right Tool Area** > **Images and Documents gallery** to an empty frame in **Center Screen Area**.

In our example we show the transition areas of the tile image as **raw image** (top left), as **fused tile image** (top right), as **shading corrected tile image** (bottom left) and finally the combination of **shading correction and fused tiles** (bottom right). In this last image no transitions between the tiles are visible any more.



12.15.6 Functions & Reference

12.15.6.1 Panorama Tool

Here you can adjust settings for the panorama acquisition.

After you have acquired the single tile images simply activate the **Perform stitching after the experiment** checkbox. If you want to stitch the images manually, click on the **Perform Stitching** button, after you have finished the experiment.

The panorama acquisition itself is performed interactively via in the **Panorama** view. If you click on '**Start Experiment**' the Panorama view will be opend and you can start to acquire the single tile images.

See also

Panorama View [457]

12.15.6.2 Panorama View

In this view you see the representation of the microscope stage. The **Live** image from the camera (blue frame) is automatically shown in the middle of the image area. Furthermore a tool window is displayed, that allows you to control the image acquisition, e.g. perform auto exposure or acquire an individual tile image.

See also

General View Options [▸ 887]

12.15.6.2.1 Stage View

In the image area the full travel range of the microscope stage is displayed. You can control the stage view using the arrow icons at the edges of the image area. The view can be enlarged, reduced or moved using the general control elements.

Navigator frame

The current stage position is shown as a tile outlined in blue, the Navigator frame. In the Navigator frame you can see the camera's live image.

To move the frame, double-click on the position on the microscope stage to which you want to move it.

To acquire images, use the **Acquisition** buttons in the **Tools window.**

See also

Tools window [▸ 457]

12.15.6.2.2 Tools window

The tool window for panorama view is normally visible in the lower right corner of the center screen area. It becomes active, if you move the cursor over it. You can use it to set acquisition parameters and acquire tile images for your panorama image.

Parameter	Description
Center to Live Navigator	Centers the stage view at the current position of the Navigator frame.
Action Buttons	With the three action buttons (**Live**, **Set Exposure**, **Continuous**) you're able to control acquisition parameters like you are used to do it on the **Acquisition** tab.
Acquire Tile Image	Acquires a tile image. This comprises all activated channels as well as Z-stacks. After the acquisition the tile image is placed in the corresponding location in the stage view.

12.16 Physiology (Dynamics)

Using the **MeanROI** offline functions you can specify user-defined measurement regions (ROIs) after acquisition of your time lapse experiment and analyze their time-dependent changes in intensity. You can display the intensity curves in charts or export the values in the form of tables. This basic functionality is available for time series images and time series with z-stack images opened in the software (excluded ZEN lite), or multi-positions (scenes) where a full time series is collected sequentially at each position (Full time series per tile Region).

The **Physiology (Dynamics)** module expands the MeanROI offline functions to give you the option of calculating online/offline (during/post acquisition) ratios and makes additional display layouts, and analysis functions available (**Timeline** view, ROI tracing etc.). To open the MeanROI view, see the following instruction:

Prerequisite ✓ You have acquired a time series experiment with one or more channels. The experiment is open and the first time point is displayed in the **2D view**.

1. Select the **MeanROI** tab from the image view tabs in the **Center Screen Area**.
   → The **MeanROI** view opens.

You are now prepared to start working with the **MeanROI** view. The following chapters will show you the first steps.

See also

Acquiring Time Series Images [▸ 49]

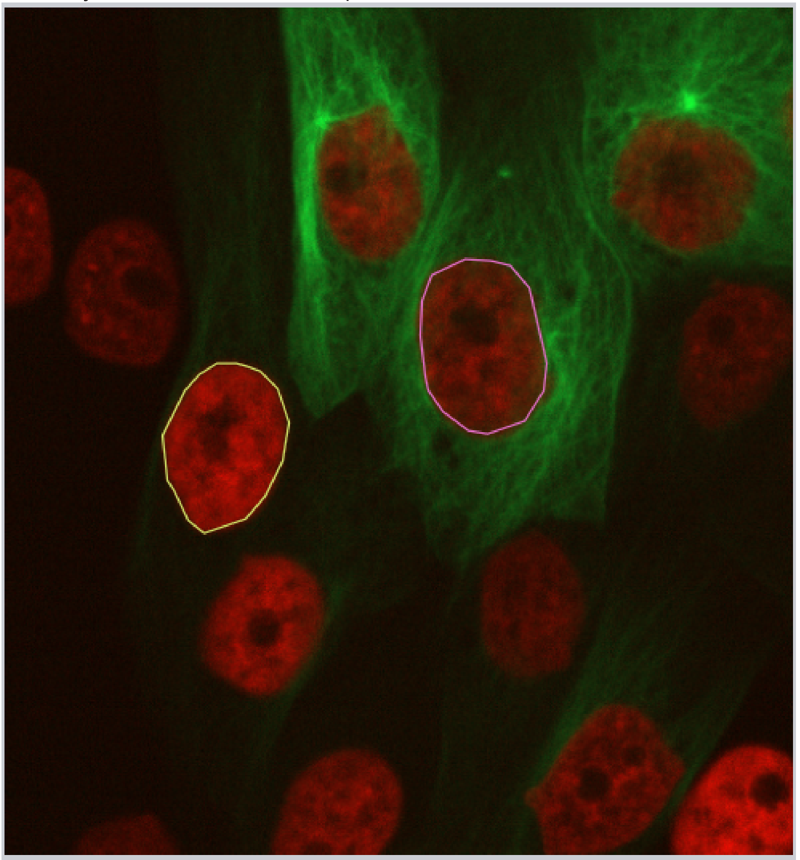
12.16.1 Workflow MeanROI View (offline)

12.16.1.1 Drawing in ROIs

Prerequisite ✓ You are in the **MeanROI View** or in the **MeanROI Setup** on **Acquisition** tab.

1. Go to the **Graphics** tab in the **View Options**.
2. Select a tool for drawing in **ROIs**, e.g. the **Polygon** tool.
3. Activate the **Keep tool** checkbox.
   → The selected tool remains active after you have drawn in an **ROI**. This means you can draw in several ROIs without having to re-select the tool.

4. Using the selected tool, in the image view draw in the objects or regions (ROIs) for which intensity measurements are required.



→ The **ROIs** are displayed in the list (Annotations/ Measurements Layer) on the **Graphics** tab.

→ Intensity measurements are performed for each **ROI** and displayed in the chart area to the right of the image view.

You have successfully defined measurement regions for the intensity measurement.

NOTICE

Measurement time

Note that the time taken to initially create measurements will vary as some date is cached to memory. Thus, when a long time series image is opened that already contains ROIs, you might have to wait briefly until ZEN completes its measurements. The duration depends on e.g. number of ROIs, number of time points, image size, number of pixels etc.

12.16.1.2 Adjusting ROIs for Time Points

If objects move laterally in the course of the time series, you can adjust the **ROIs** at each **Time Point** in order to follow the objects.

Prerequisite

✓ You have defined at least one ROI.

✓ You are in the **MeanROI** view.

1. Open the **Dimensions** tab in the general view options.
2. Use the **Time slider** to scroll through the time points. Stop at the first time point at which you want to adjust a ROI.

3. Open the **ROI Tracing** tab and activate the **Enable ROI Tracing** checkbox. To edit the position of a single **Key frame** change the **Key Frame Edit Mode** to single mode . Note that you can only select the key frame edit mode if the frame number is set to a value >1.
4. Adjust the position of the ROI using drag & drop. To do this, select the ROI in the image area by pressing the left mouse button. Then move the ROI to the new position and release the mouse button. Note that rectangular or contour ROIs can also be rotated.
5. If necessary, you can change the shape of a ROI, by right-clicking on a ROI and selecting **Edit Points** (e.g. for polygon contours). It is also possible to rotate a ROI if necessary. Note that if the area of the ROI changes, the mean intensity value will change. For ratio values in which thresholding is applied, only "valid" pixels will be respected (see *Basics of Calculation of Intensity and Ratio Values [▸ 463]*). Ratios can only be performed if you have the module **Physiology (Dynamics)**.
6. Adjust the shape/ rotation of the ROI, by drag & drop the contour points/ via the rotation handle.
   → Changes to the position and shape of the ROIs are adopted for all subsequent time points.
7. Repeat the previous steps for all other time points for which you want to adjust an ROI. For a selected ROI you can see a list of the time points in which its position/shape was modified. As the distance (in frames) between each key frame can vary, a linear interpolation is used to smoothly progress the ROI through the time points. Alternatively, deactivate this (constant) or set it to a spline method that may better describe the progression of the object you are tracing.

You have successfully adjusted the measurement regions to the course of the experiment.

12.16.1.3 Adjusting the Display

Here you will find out how to adjust the display of the measured intensity values in charts and tables according to your wishes.

Prerequisite
- ✓ You are in the **MeanROI** view or **MeanROI Setup**.
- ✓ You have defined at least one ROI.

1. Select the **Layout** tab in the view options.
2. To adjust the layout of the image and diagram display, select the desired display mode under **Image and Chart**.
3. If you also want your data to be displayed in table form (note for MeanROI setup the table is only displayed after the acquisition is completed), select the desired display mode under **Image and Chart with Table**. With the **Export** tab, you can export the data table directly as a comma separated values file or create a separate data table document. Tables created in this manner contain additional information, for details see *Basics of Calculation of Intensity and Ratio Values [▸ 463]*. The table in the **MeanROI** view only shows two values per ROI and time point, the mean intensity of pixels above the set threshold per channel and the mean ratio value derived from (common) "valid" pixels.
4. In the **MeanROI** view (not the MeanROI setup) you can interact with a table in various ways. See *Interaction with a table in MeanROI view [▸ 464]*.
5. You can determine the zoom (range) of the charts of all given channels (including Ratio charts) by clicking on the chart, selecting it, and scrolling with the mouse wheel.
6. For **Offline Analysis** only: Select a suitable layout for the image, chart and table display.
7. If you want to adjust the axis scaling (range), go to the **Charts** tab in the view options.
8. To define the minimum and maximum values of the axes manually, click on the **Fixed** button under **X-/Y-Axis**.
   → The **Min** and **Max** input fields for the axis are activated.

9. Enter the desired values first into the **Max** and then the **Min** input fields.
   → The minimum and maximum axis values of the diagrams are adjusted. Note that the Y-axis scaling can be adjusted individually for each chart.
10. To change the unit of the X axis, click on the **Fixed** button under **X Units**.
   → The dropdown menu for the units is activated. You can now select the desired unit.
11. On the **Layout** tab, it is also possible to determine if a given channel view and/ or chart panel should be hidden. This is useful if these do not contain information that needs to be visible, thus increasing space on the screen for the remaining items. For example, in many applications transmitted light is used to monitor the specimen, but the intensity information (chart) is not required. The controls behave in a similar manner to the channel toggles on **Dimensions** tab.

You have successfully adjusted the display of the intensity values.

12.16.1.4 Using Background Correction

Use this function to subtract background values from the measurement values. A background correction will allow you to make a better comparison of the magnitude of any fluorescent intensity changes observed over the time course of an experiment. Determine the background value with the help of a Background ROI or define a fixed value. Note that the background correction for ROI is only available if there are at least two ROI defined in the image!
If the ratio calculations are enabled, the background correction parameters are defined on the **Ratio** tab. The background correction values on **MeanROI** tab are disabled, i.e. not used in this case.

Defining a Background ROI

Prerequisite ✓ You are in the **MeanROI** view or **MeanROI** setup.

1. At the desired time point of the time series, draw a **ROI** into a part of the image that contains only background signal in all channels.
2. Go to the **MeanROI** tab > **Background Correction** section and activate the radio button **ROI**. Note that this function is only available if two or more ROIs are present.
3. In the drop down, select the ROI-ID of the ROI that you defined in the first step.
4. To edit the **Background ROI**, simply draw a new ROI and select it from the dropdown list.

You have successfully defined a **Background ROI**. The mean intensity of the background ROI is subtracted from the measured values of the **ROIs** in a channel- and time-point-specific manner. The corrected values are adopted into all diagrams and tables.

Defining a fixed background value

Prerequisite ✓ You are in the **MeanROI** View or **MeanROI** Setup.

1. On the **MeanROI** tab in the **Background Correction** section select the **Constant** option.
   → The associated input field is activated.
2. Enter a fixed background value into the **Constant** input field.
3. Press *Enter* to update the measurements.

The defined background value is subtracted from all measured values of the **ROIs** in a time-point-specific manner.

12.16.1.5 Exporting a Data Table

Prerequisite
- ✓ You are in the **MeanROI** view.
- ✓ You have defined at least one ROI.

1. Select the **Export** tab in the View Options.
2. In the **Data Table** section click on the **Save As (*.csv)** button.
   → The **Save As** dialog opens.
3. Enter a suitable file name, navigate to the desired folder and click on **Save**.
   → **All** the measurement data are saved as comma-separated values in a csv file. This contains the time information, marker events, the geometric area of the ROIs, the area of ROI adjusted by threshold (only with **Physiology (Dynamics)** module), the mean intensity of the ROI, the mean intensity of the ROI adjusted by threshold (only with **Physiology (Dynamics)** module), mean ratio value (only with **Physiology (Dynamics)** module), focus position, and incubation events/ values (if configured) for each channel and each ROI.

12.16.1.6 Calculating a Ratio for One Wavelength

Prerequisite
- ✓ To calculate ratios (quotient of two fluorescence intensities) and display ratio images, you need the **Physiology (Dynamics)** module.
- ✓ You have a suitable image data set open.
- ✓ You are in the **MeanROI** view on the **Ratio** tab (view option).

1. Activate the checkbox **Enable Ratio Calculation**.
2. In the **Method** dropdown list, select the **Single Wavelength (F/F_0)** entry.
3. In the **Calculation** dropdown list select the channel for calculating the ratio.
4. In the Reference image (Ft_0) setup, define the frames of the time series image from which you want the reference value Ft_0 to be calculated.
5. Click on the **Update** button.
   → The ratio values are calculated. The ratio image and a diagram for the ratio values are displayed in the **MeanROI** view. For very large images (pixels and time points) it might be necessary to use the **Cache Ratio Image** function on the **Ratio** tab, as this will eliminate flickering when playing through the images at speed.

You have successfully calculated a ratio for a single wavelength dye such as Fluo-4.

12.16.1.7 Calculating a Ratio for Two Wavelengths

Prerequisite
- ✓ To calculate ratios (quotient of two fluorescence intensities) and display ratio images, you need the **Physiology (Dynamics)** module.
- ✓ You have a suitable image date set open.
- ✓ You are in the **MeanROI View** on the **Ratio** tab (view option).

1. In the **Method** dropdown list select the **Dual Wavelength** or any other of the Ratio Type formulas present. entry.
2. In the **Calculation** dropdown lists select the channels for calculating the ratio.
   → The ratio values are calculated automatically.
3. Click on the **Cache ratio image** button to cache all the ratio images of the current time series with the given ratio calculation parameters.

→ The ratio values are calculated. The ratio image and a ratio diagram are displayed on the **MeanROI** view. For very large images (pixels and time points) it might be necessary to use the **Cache Ratio Image** function on the **Ratio** tab, as this will eliminate flickering when playing through the images at speed.

You have successfully calculated a ratio for a dual wavelength dye such as Fura-2.

12.16.1.8 Basics of Calculation of Intensity and Ratio Values

The ratio calculation in ZEN blue MeanROI / Physiology functions in the following manner:

After you have set up the ratio to your satisfaction (background correction/ thresholding), you can gather/ view all the results using the export functions found on the export tab in **MeanROI** view. You can also view a smaller table within the **MeanROI** view itself by activating the appropriate layout.

How are your threshold and background values handled for intensity and ratio measurements? The threshold value will be applied prior to background subtraction if both are active. If this is this is the case, then for charts/tables the intensity values of any given ROI are handled this way: if a pixel in the ROI is under the threshold value, it will be ignored for the calculation of the mean value of the ROI. After that the background is subtracted from the mean value to get the corrected intensity value of the ROI (note if no pixel remains valid in the ROI after the application of the threshold, the corrected mean intensity is always 0 and hence the ratio is also 0). In the case of the ratio image, each pixel is "validated" based on the threshold value. If the pixel is above the threshold then the pixel value is kept (i.e. is valid), otherwise it is set as NaN (Not a Number, which is not the same as zero) and is considered invalid). As before, background correction is done after threshold. If the pixel value is NaN, the ratio pixel value is NaN. If the pixel is still valid then use pixel value - background value in the ratio calculation. If a negative value results, it is clipped to 0.

The following example shows how a ratio value is generated based on the applied background and thresholding values:

Consider a region of interest that is 6 pixels wide by 1 pixel high. The pixels of the region in the Wavelength 1 image are as follows:
[50, 75, 100, 125, 150, 175].

For the purpose of this example, assume the threshold = 60 for wavelength 1.

ZEN thresholds Wavelength 1 to obtain:
[--, 75, 100, 125, 150, 175].

The pixels of the region in the Wavelength 2 image are as follows:
[25, 25, 25, 25, 100, 100].

For the purpose of the example, assume the threshold = 50. ZEN thresholds Wavelength 2 to obtain:
[---, ---, --- , ---, 100, 100].

ZEN computes the Ratio by only rationing the averaged values for the valid pixels in each individual wavelength. To recap, the pixels for each wavelength were:

[--, 75, 100, 125, 150, 175] (Wavelength 1)
[---, ---, --- , ---, 100, 100] (Wavelength 2)

The ratio value of this region is calculated by taking into account only the common area, which corresponds to the area of the sum of the valid pixels common to the two wavelengths (which in this example is only 2 “valid” pixels).

Using the threshold values as above, the pixels that are used to calculate the ratio average are:
150/100
175/100
which gives a ratio of 1.625.

To get an overview of all the results, including the original values not corrected for there validity in this manner, use the data table creation function in the MeanROI export tab. This opens, for example, a new document or allows you to export the results as a *.csv file. This data table/ *.csv includes for each ROI the following information /measurements:

Value in ID in table header	Description
`<Channel name>`_`<Region ID>`_Area	Geometric area of ROI (constant for both channels).
`<Channel name>`_`<Region ID>`_IntensityAreaThrs	Threshold corrected area within ROI for given channel.
`<Channel name>`_`<Region ID>`_IntensityMean	Mean intensity of pixels within the geometric area of the ROI.
`<Channel name>`_`<Region ID>`_IntensityMeanThrs	Mean intensity of pixels above the set threshold for the given channel.
Ratio `<Region ID>`	Mean Ratio value derived from common “valid” pixels.

This is repeated for the second channel, and at the very end you will find the Ratio value. Thus, the threshold corrected values are provided for each channel (mean intensity and the corresponding area from which this is derived) as well as the ratio value for the common valid pixels. Relative time, markers, focus values and parameters from the incubation (if configured) are also listed. In the embedded table in Mean ROI view you will find a summary that gives the following values: Mean intensity of pixels above the set threshold for each channel (wavelength) and the corresponding ratio values. No Area values are given here.

12.16.1.9 Interaction with a table in MeanROI view

In the **MeanROI** view (not the MeanROI setup) you can interact with a table in the following ways:

- Scroll up or down or left to right. Time values are given at the far left and at the far right any markers are shown at the time point they were created. In between you find the values for each ROI in the first channel, then the second and so on, then the ratio values.
- If you click on the column header you select the entire column and in your charts you will see the trace corresponding to this ROI is highlighted by a thicker line. Multiple columns can be selected by pressing and holding shift key.
- If you click any given value in a column, not only will the trace of the corresponding ROI be highlighted, the images that correspond to this time point will be displayed, and the playhead (vertical blue line) of all charts will synchronise to this time point. This allows quick and easy examination of the data/ events.

12.16.2 Workflow Physiology (Dynamics) Experiments

If you own the **Physiology (Dynamics)** module you can use the **MeanROI** setup to specify user-defined measurement regions (ROIs) before the acquisition of your time lapse experiment and analyze their time-dependent changes in intensity online during acquisition. Ratios can also be calculated and displayed online - these are the typical functions used in physiology/ calcium Fura-2 applications.

Before the experiment

A precondition for a physiology experiment is a **Time Series** experiment (which can include a z-stack acquisition), which is set up in the **Time Series** tool. Adding a time dimension to your experiment allows you to activate the optional tool **Dynamics**. This tool contains the button for opening **MeanROI Setup**. Here you can draw in ROIs and adjust the display layout of the measurement results. Note that when the setup is opened a snap is automatically acquired, on the basis of which you can configure the settings for the subsequent experiment, like for example the ratio parameters. The structure of the **MeanROI** setup is based on the *MeanROI view [▸ 859]*, making it easier to learn. Note that online measurements on tiles and positions experiments are not possible, but you can perform measurements on tiles or position data collected over time (i.e. multi-scene time series) in **MeanROI** view post acquisition.

During the experiment

After being started, physiology experiments are displayed in the online mode of the **MeanROI View**. This allows you to analyze and follow the experiment during acquisition. The structure and options largely correspond to the offline mode of the **MeanROI View**. We therefore recommend that you familiarize yourself with the *MeanROI View (offline) [▸ 458]* before performing your Physiology experiment.

After the experiment

After you have performed your Physiology experiment the data are displayed in the offline mode of the **MeanROI** view and can be analyzed, processed and exported there. For more information, see also *Workflow MeanROI View (offline) [▸ 458]*.

Prerequisite

- ✓ To perform physiology experiments, you need the **Physiology (Dynamics)** module.
- ✓ You have *created a new experiment [▸ 44]*, *defined at least one channel [▸ 43]* and adjusted the focus and exposure time.
- ✓ You are on the **Acquisition** tab.

1. Activate the **Time Series** tool in the **Acquisition Dimensions** section.
   → The **Time Series** tool appears in the **Left Tool Area** under **Multidimensional Acquisition**.
2. Enable the **Dynamics** tool in the experiment manager.
   → The **Dynamics** tool now appears in the in the **Left Tool Area** under **Applications**.
   → Note that the tool is not available if the **Tiles** or **Panorama** dimensions are activated. Deactivate these dimensions to make the tool available.
3. Set up a *time series experiment [▸ 49]*.
4. Open the **Dynamics** tool.
5. Click on the **MeanROI Setup** button.

You have completed the general prerequisites for Physiology experiments.

12.16.2.1 Setting up an Experiment in MeanROI Setup

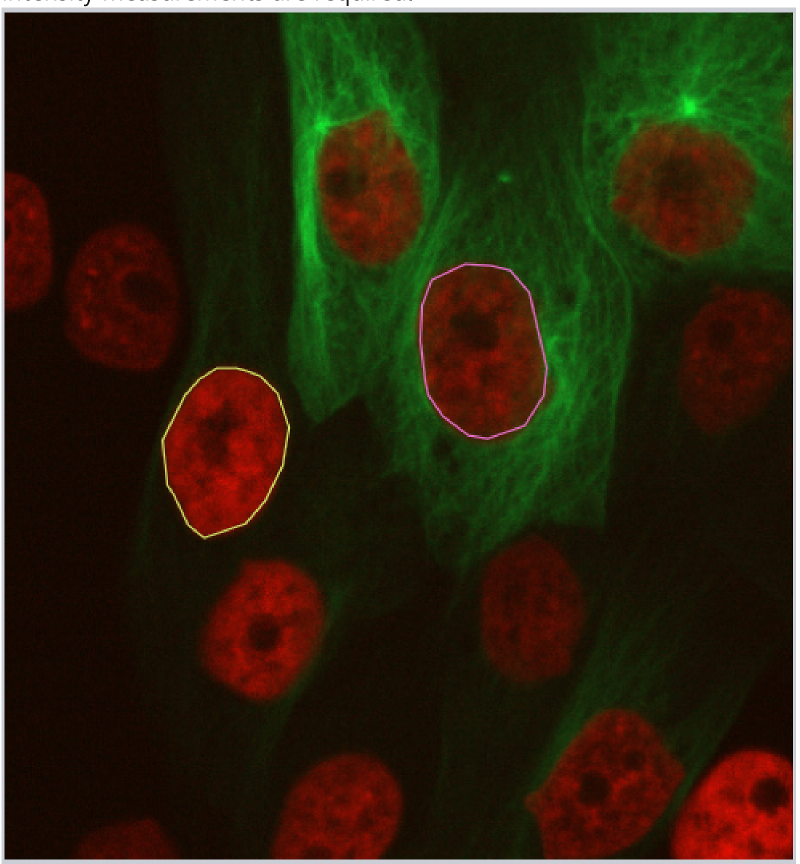
Prerequisite ✓ You have read the *Workflow Physiology (Dynamics) Experiments [**▸ 465**]* chapter.

1. Activate the **Dynamics** checkbox in the Experiment Manager.
2. In the **Dynamics** tool, click on the **MeanROI Setup** button.
   → MeanROI setup opens.
   → An image is acquired automatically, on the basis of which you can configure your settings. You can click on **Snap** at any time to update the image.

12.16.2.1.1 Drawing in ROIs

Prerequisite ✓ You are in the **MeanROI View** or in the **MeanROI Setup** on **Acquisition** tab.

1. Go to the **Graphics** tab in the **View Options**.
2. Select a tool for drawing in **ROIs**, e.g. the **Polygon** tool.
3. Activate the **Keep tool** checkbox.
   → The selected tool remains active after you have drawn in an **ROI**. This means you can draw in several ROIs without having to re-select the tool.
4. Using the selected tool, in the image view draw in the objects or regions (ROIs) for which intensity measurements are required.



   → The **ROIs** are displayed in the list (Annotations/ Measurements Layer) on the **Graphics** tab.
   → Intensity measurements are performed for each **ROI** and displayed in the chart area to the right of the image view.

You have successfully defined measurement regions for the intensity measurement.

NOTICE

Measurement time

Note that the time taken to initially create measurements will vary as some date is cached to memory. Thus, when a long time series image is opened that already contains ROIs, you might have to wait briefly until ZEN completes its measurements. The duration depends on e.g. number of ROIs, number of time points, image size, number of pixels etc.

12.16.2.1.2 Adjusting the Display

Here you will find out how to adjust the display of the measured intensity values in charts and tables according to your wishes.

Prerequisite

- ✓ You are in the **MeanROI** view or **MeanROI Setup**.
- ✓ You have defined at least one ROI.

1. Select the **Layout** tab in the view options.
2. To adjust the layout of the image and diagram display, select the desired display mode under **Image and Chart**.
3. If you also want your data to be displayed in table form (note for MeanROI setup the table is only displayed after the acquisition is completed), select the desired display mode under **Image and Chart with Table**. With the **Export** tab, you can export the data table directly as a comma separated values file or create a separate data table document.
   Tables created in this manner contain additional information, for details see *Basics of Calculation of Intensity and Ratio Values [**▸ 463]*. The table in the **MeanROI** view only shows two values per ROI and time point, the mean intensity of pixels above the set threshold per channel and the mean ratio value derived from (common) "valid" pixels.
4. In the **MeanROI** view (not the MeanROI setup) you can interact with a table in various ways. See *Interaction with a table in MeanROI view [**▸ 464]*.
5. You can determine the zoom (range) of the charts of all given channels (including Ratio charts) by clicking on the chart, selecting it, and scrolling with the mouse wheel.
6. For **Offline Analysis** only: Select a suitable layout for the image, chart and table display.
7. If you want to adjust the axis scaling (range), go to the **Charts** tab in the view options.
8. To define the minimum and maximum values of the axes manually, click on the **Fixed** button under **X-/Y-Axis**.
   → The **Min** and **Max** input fields for the axis are activated.
9. Enter the desired values first into the **Max** and then the **Min** input fields.
   → The minimum and maximum axis values of the diagrams are adjusted. Note that the Y-axis scaling can be adjusted individually for each chart.
10. To change the unit of the X axis, click on the **Fixed** button under **X Units**.
    → The dropdown menu for the units is activated. You can now select the desired unit.
11. On the **Layout** tab, it is also possible to determine if a given channel view and/ or chart panel should be hidden. This is useful if these do not contain information that needs to be visible, thus increasing space on the screen for the remaining items. For example, in many applications transmitted light is used to monitor the specimen, but the intensity information (chart) is not required. The controls behave in a similar manner to the channel toggles on **Dimensions** tab.

You have successfully adjusted the display of the intensity values.

12.16.2.1.3 Using Background Correction

Use this function to subtract background values from the measurement values. A background correction will allow you to make a better comparison of the magnitude of any fluorescent intensity changes observed over the time course of an experiment. Determine the background value with the help of a Background ROI or define a fixed value. Note that the background correction for ROI is only available if there are at least two ROI defined in the image!
If the ratio calculations are enabled, the background correction parameters are defined on the **Ratio** tab. The background correction values on **MeanROI** tab are disabled, i.e. not used in this case.

Defining a Background ROI

Prerequisite ✓ You are in the **MeanROI** view or **MeanROI** setup.

1. At the desired time point of the time series, draw a **ROI** into a part of the image that contains only background signal in all channels.
2. Go to the **MeanROI** tab > **Background Correction** section and activate the radio button **ROI**. Note that this function is only available if two or more ROIs are present.
3. In the drop down, select the ROI-ID of the ROI that you defined in the first step.
4. To edit the **Background ROI**, simply draw a new ROI and select it from the dropdown list.

You have successfully defined a **Background ROI**. The mean intensity of the background ROI is subtracted from the measured values of the **ROIs** in a channel- and time-point-specific manner. The corrected values are adopted into all diagrams and tables.

Defining a fixed background value

Prerequisite ✓ You are in the **MeanROI** View or **MeanROI** Setup.

1. On the **MeanROI** tab in the **Background Correction** section select the **Constant** option.
   → The associated input field is activated.
2. Enter a fixed background value into the **Constant** input field.
3. Press *Enter* to update the measurements.

The defined background value is subtracted from all measured values of the **ROIs** in a time-point-specific manner.

12.16.2.2 Starting and Influencing an Experiment

Prerequisite ✓ You have read the *Workflow Physiology (Dynamics) Experiments [▸ 465]* chapter and set up an experiment in **MeanROI Setup**.

✓ You are on the **Acquisition** tab.

1. Start your Physiology experiment by clicking on the **Start Experiment** button.
   → The time series experiment is started. The *MeanROI View [▸ 859]* (online) opens and displays the current images and the intensity curves for each ROI measured online. The intensity curves are displayed in the Time Line View and in the diagrams. Note that the **MeanROI** view will display at the third time point. This is noticeable when the interval time is longer. Thus this display delay should fall into the typically base line of this type of experiments, i.e. prior to the first stimulus of the sample.
2. You can pause the experiment at any time by clicking on the **Pause Experiment** button and continue it again by clicking on the **Continue Experiment** button.

3. The **Focus** can be adjusted during the experiment. To prevent images that are not sharp being acquired, pause your experiment and use the **Live** acquisition button to adjust the focus. Then continue the experiment. Note that using the Live view only works with experiments run in interactive mode. In triggered acquisition scenarios this is not possible.
4. Adjust the display of the intensity values during the experiment by changing the settings on the **Layout or Charts** tab. The unit of the X-axis cannot be changed during the experiment.
5. You can move and change ROIs during acquisition. The changes are adopted for all time points, see Drawing in and adjusting ROIs. Note that ROI tracing functions (these allow objects to be followed in XY) are only available after an acquisition.
6. Activate **Switches** in the **Time Series** tool during the experiment to perform the corresponding actions.
   → Various events, such as the activation of switches or the pausing of the experiment, are labeled in the Time Line view by markers.
7. On the **Dimensions** tab deactivate the **Follow Acquisition** checkbox to analyze the data acquired up to that point. To do this, select the corresponding time points using the **Time** slider, the diagram sliders or the Time Line view slider in the **MeanROI** view.
8. Change the size of the area marked in blue in the Time Line View to adjust the section displayed in the charts (time axis).

You have successfully started the experiment, analyzed it online and influenced it.

12.16.2.2.1 Adjusting ROIs during experiments

If objects move laterally in the course of the experiment, you can adjust the **ROIs** at any time during the experiment in order to follow the objects.

Prerequisite
- ✓ You have defined at least one ROI.
- ✓ You have started your Physiology experiment.

1. In the **Experiment Manger** click on **Pause experiment** button.
2. Adjust the position of the **ROI** using drag & drop. To do this, select the ROI in the **image area** by left-clicking and hold the mouse button down. Then move the ROI to the new position and release the mouse button.
3. To change the shape of an **ROI** left click on an ROI and drag the bounds to adjust the size.
   → Changes to the position and shape of the **ROIs** are adopted for all time points.
4. Repeat the previous steps for all subsequent ROIs that you wish to adjust. Note that you can select multiple ROIs and adjust all the positions simultaneously.

You have successfully adjusted the measurement regions (ROIs) to the course of the experiment.

12.16.3 Sample Experiment Fura-2 with DG4/5

12.16.3.1 Step 1: Creating channels

Prerequisite
- ✓ To perform the experiment, you need the **Physiology (Dynamics)** module.
- ✓ You have a **Sutter DG4/5** with appropriate excitation filters for **Fura-2** and a **Fura-2 filter set** in the microscope's reflector wheel.
- ✓ You are on the **Acquisition** tab.

1. Create a new experiment in the **Experiment Manager**, e.g. "**Physiology Fura-2**".
2. Add the channel Fura-2 using **Smart Setup**.
3. Activate the **Time Series** checkbox in the acquisition dimensions.

4. Open the **Channels** tool.
5. Select the Fura-2 channel from the list.
6. Click on **Options** and select **Duplicate**.
7. Select the first Fura-2 channel from the list.
8. Click on the **Options** and select **Rename**.
   → You can now rename the channel, e.g. **Fura-2 340 nm**.
9. Repeat steps 7 and 8 to rename the second channel, e.g. **Fura-2 380 nm**.
10. Select the **Fura-2 380 nm** channel.
11. Select another LUT from the dropdown list, e.g. red.
12. Select the entry **21 HE Ex. FURA 380** from the Excitation dropdown list.
    → The excitation filter is used for this channel.
13. Adjust the exposure time and focus for both channels.

You have created the channels for your experiment.

12.16.3.2 Step 2: Setting up a time series and creating switches

1. Open the **Time Series** tool.
2. Using the **Duration** slider and the dropdown list for the unit, specify the duration of the experiment, e.g. 10 min.
3. Using the **Interval** slider and the dropdown list for the unit, specify the length of the interval between acquisitions, e.g. 1 second.
4. To create interactive switches open the **Interactive Switches** section in the **Time Series** tool. This section is visible only if the **Show All** mode is activated.
5. Click on the **Add** button .
   → A new switch is added.
6. Edit the switch by clicking on the arrow to the right of the switch.
   → The switch properties are visible.
7. Enter a name, e.g. **Fast**. Activate the **Color** checkbox and select a color, e.g. blue. Define an action to be performed when you activate the button, e.g. **As fast as possible**.

You have successfully set up a time series and created a switch.

12.16.3.3 Step 3: Setting up an online ratio

1. Open the **MeanROI setup** from inside the **Dynamics** tool.
   → Mean ROI definition opens and snaps of the configured channels are acquired automatically and displayed in the Center Screen Area. The diagrams for each image are displayed to the right of this.
2. Select the **Online Ratio** tab from the view options and activate live ratio generation.
3. Under **Method** select the **Dual Wavelength** entry from the dropdown list.
4. Under **Calculation** select the **Fura-2 340 nm** entry from the dropdown list in the numerator of the formula.
5. Under **Calculation** select the **Fura-2 380 nm** entry from the dropdown list in the denominator of the formula.
   → A preview of the ratio image, which is calculated according to the ratio settings, is displayed.

You have successfully activated the ratio functions and specified the calculation of the ratio.

12.16.3.4 Step 4: MeanROI Setup

1. Open the **MeanROI** setup from inside the **Dynamics** tool.
2. On the **Graphics** tab, select a tool for drawing in ROIs, e.g. Circle.
3. Activate the **Keep Tool** checkbox.
4. Draw your ROIs into one of the images.
5. Deactivate the **Keep Tool** checkbox and select the selection tool (**arrow**) again.
6. On the **Layouts** tab select a layout for the image and diagram display, e.g. multichannel image and single channel charts.
7. Go to **Charts** tab and click on the **Fixed** button under **X Units** and select a unit from the dropdown list, e.g. seconds.
8. Click on **Exit** at the top left of **MeanROI Setup**.

You have successfully configured and adjusted the MeanROI Setup.

12.16.3.5 Step 5: Starting, analyzing and influencing an experiment

1. Start the experiment by clicking on the **Start Experiment** button.
   → The experiment is started. In our example an image is acquired every second for a period of 10 minutes. The experiment opens in the online mode of the MeanROI View, which displays the current images and measurements.
2. Activate the created switch at the desired time point. To do this, open the **Switches** section in the **Time Series** tool. Click on a switch as soon as you want its action to be performed, e.g. click on the "**Fast**" switch to acquire the subsequent images as quickly as possible one after the other. A marker will mark the time point at which the switch was activated on the X axis in the color of the switch (e.g. blue).
3. Once the time series has been completed you can analyze the experiment in the offline mode of the **MeanROI** view, process it and export its values.

You have successfully performed the experiment.

12.17 Shuttle & Find

Fig. 28: SEM / LM system for correlative microscopy

This module enables you to relocate sample positions in two different microscopes, e.g. a light microscope and a scanning electron microscope (SEM). Afterwards you can correlate the two images to one merged image. This technique is called correlative microscopy or just "CorrMic". It is used to combine the two worlds of scanning electron microscopy and light microscopy and brings it together in one image.

The samples can be mounted in special designed correlative holder systems (with three correlative calibration markers) from ZEISS. Also user-defined holder systems with three calibration markers can be used. Biological samples are mainly deposited on cover glasses or on TEM grids. In contrast to biological samples, the shape and size of material samples vary strongly. In respect to these requirements, the correlative holders were designed accordingly.

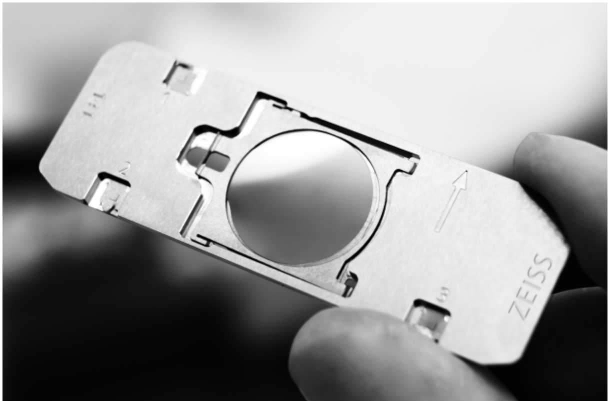


Fig. 29: Example of a correlative ZEISS sample holder

12.17.1 Settings and Image Acquisition with the Light Microscope

Before acquiring an image with the light microscope and using it for correlative microscopy, it is necessary to make general settings e.g. stage calibration, camera orientation, calibrating objectives and setting the correct scaling. Please notice that we do not describe all theses topics within this guide as we focus on the Shuttle & Find workflow only.

Furthermore we will not describe basic functionality of the software in this guide, like program layout or general image acquisition topics.

See also

Shuttle & Find Sample Positions at the Electron Microscope [480]

12.17.1.1 Mounting the Sample Holder to the LM

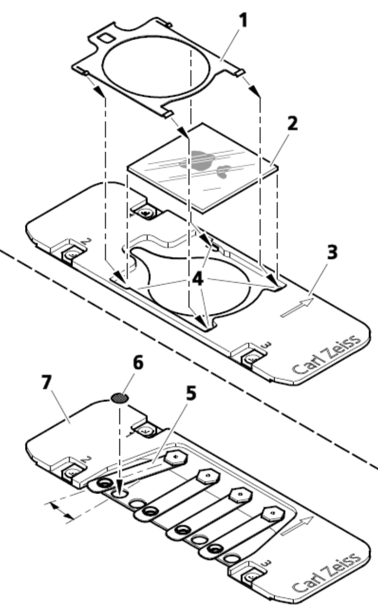


Fig. 30: Mounting of cover glasses or TEM grids

1. Place the cover glass **(2)** in the suitable sample holder and fix it.

   In case of using the holder **Life Science Cover Glass 22x22**:

   – Remove the clamping frame **(1)** using tweezers.
   – Insert the cover glass **(2)** in the sample holder **(3)**.
   – Slide in the clamping frame into the sample holder until the clamps are clicking into place **(4)**.

   In case of using the holder **Life Science for TEM grids**:

   – Lift the spring of the appropriate position and turn it sidewards **(5)**.
   – Insert the TEM grid **(6)** into the provided holding spot of the holder and fix it with the spring **(7)**.

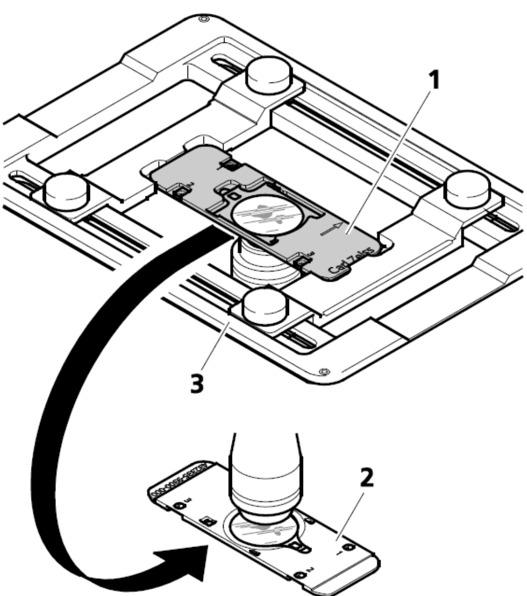


Fig. 31: Inserting a sample holder

2. Insert the sample holder **(1)** into the mounting frame of the microscope stage in the following way:
   - For inverted stands, see **(3)**.
   - For upright stands, see **(2)**.

12.17.1.2 Starting the LM Software

For correlative microscopy with light microscopes ZEN (blue edition) software has to be installed. In addition you need to licence the **Shuttle & Find** modul.

1. To start the software click on the corresponding program icon on your desktop.
   → The application selection window will appear.
2. Click on **ZEN pro** or **ZEN system** to start the desired application.
   → The software will start now. Make sure that you have activated the **Shuttle & Find** module in the menu **Tools > Modules Manager ...**
3. In the **Left Tool Area** switch to the **Acquisition** tab and activate **Shuttle & Find**.
4. Open the **Shuttle & Find** tool.

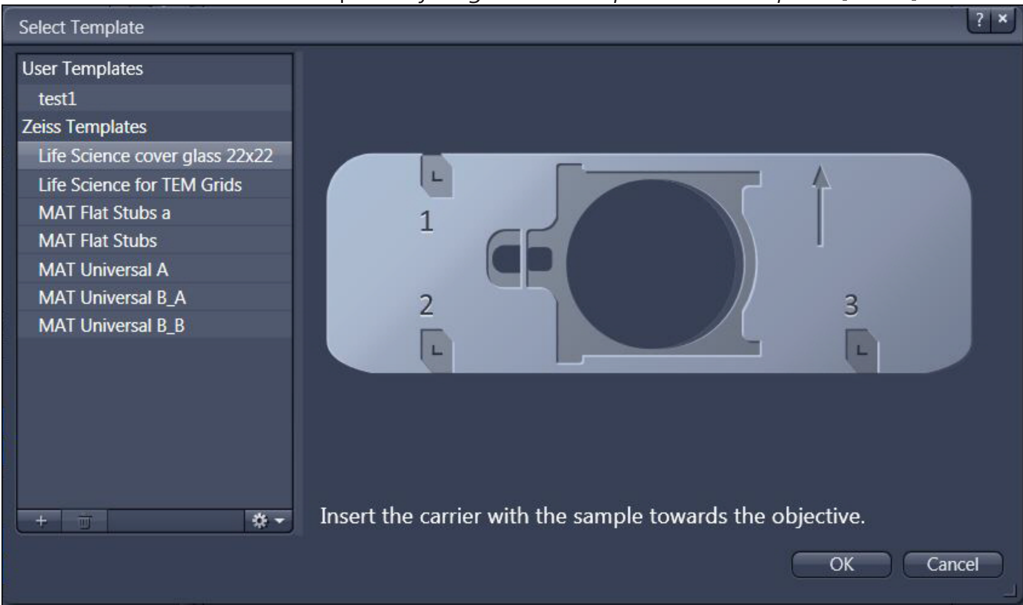
You have successfully started the software. Now you can start working with the **Shuttle & Find** module.

12.17.1.3 Selecting the Sample Holder

Prerequisite
- ✓ You have activated **Shuttle & Find** in the **Experiment Manager**.
- ✓ You are in the **Shuttle & Find** tool.

1. Click on the **Select…** button to open the **Select Template** dialog and to choose the correlative holder you want to use. Different types of correlative holders are available, see Appendix Correlative Sample Holders

2. In the **Select Template** dialog select the correlative holder you want to work with. If you want use your own sample holders, click on the **Add** button below the list and follow the instructions in the chapter *Defining a new sample holder template [▸ 475]*.



3. Click on the **Ok** button to close the dialog.

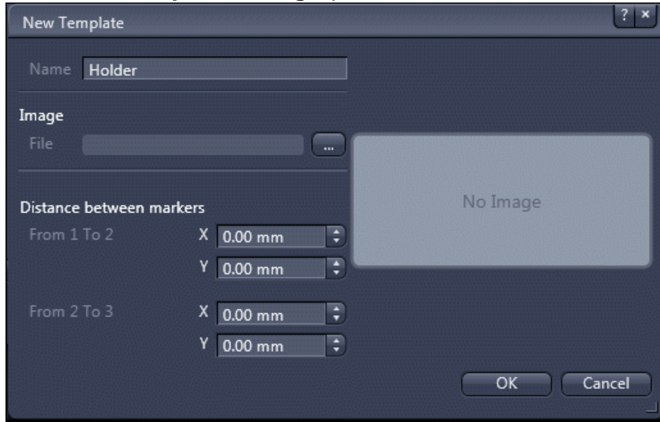
You can now continue with the calibration of the sample holder, like it is shown in the chapter *Calibrating the sample holder [▸ 476]*. The calibration of the sample holder is mandatory to acquire images.

12.17.1.4 Defining a New Sample Holder Template

With this dialog you can define new correlative holders in addition to the existing holder templates. It is not mandatory to use correlative holders from ZEISS. User-defined correlative holders with 3 fiducial markers can be used as well.

1. To open the dialog click on **Add** in the **Select Template** dialog. This dialog can be opened via the **Shuttle & Find** tool.

→ The **New Template** dialog opens.



2. Type in a name for the new holder / sample carrier. An image of the new holder can be loaded as well.
3. Insert the distances (in millimeters) between the first and the second marker and between the second and third marker.

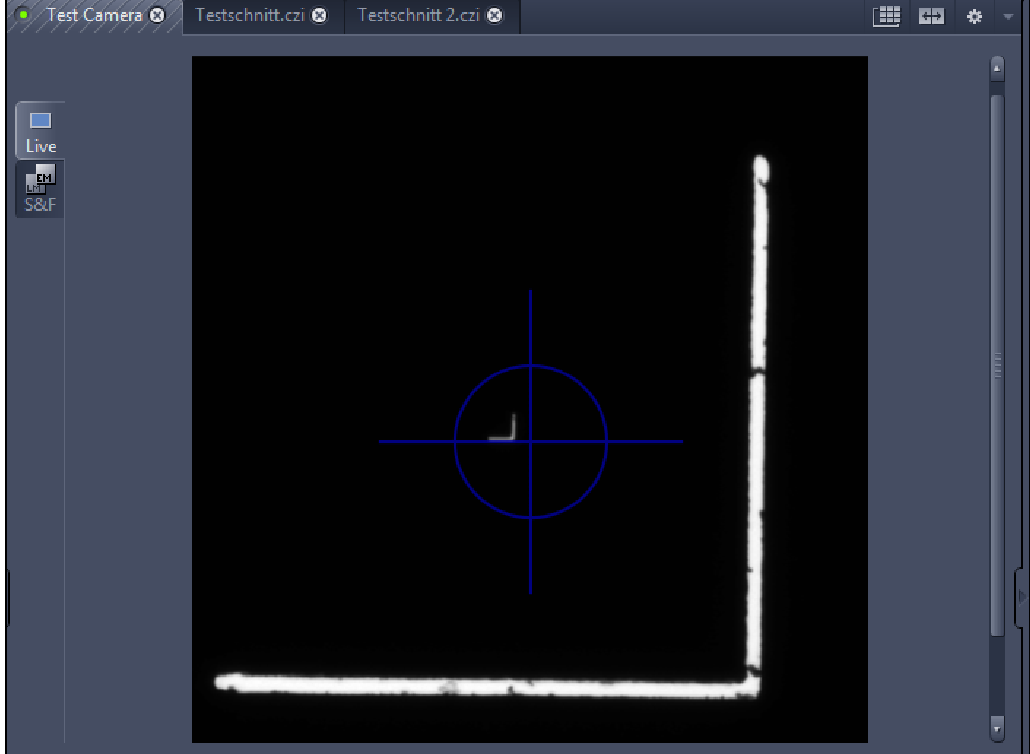
- → The distances can be determined using the **Stage Control** dialog accessible via the **Light Path** tool in **Right Tool Area** tab. We recommend to do this before you start the New template dialog. Write down the distances to be prepared to enter them within the New Template dialog.
- → Activate the live view in the Center Screen Area by clicking on the **Live** button in the Locate tab.
- → Navigate the stage manually to the calibration marker on the sample holder by means of the joystick and note the x/y-coordinates of the marker.
- → Repeat this procedure for all three markers and calculate the distances between marker 1 and marker 2 and between marker 2 and marker 3, respectively.

12.17.1.5 Calibrating the Sample Holder

Correlative sample holders have three fiducial markers enabling a three point calibration (signed with the numbers 1-2-3) The calibration markers consist of one small (length 50 µm) and a large L-shape marker (length 1 mm). The bigger marker is used for coarse orientation, whereas the smaller marker is used for the calibration.

12.17.1.5.1 Preparing Calibration

1. Click on **Live** in the **Acquisition** tab to activate the live view in the **Center Screen Area**.
2. Navigate the stage manually to the first calibration marker on the sample holder (marked with No. 1) by means of the joystick. It is enough if you move the stage to the larger L-shaped calibration marker. The smaller marker will be detected automatically within the **Sample Holder Calibration Wizard**.To locate the marker positions we recommend to use a dry objective with low magnification (5x – 20x).



3. Open the **Shuttle & Find** tool.
4. Click on **Calibrate…** to open the **Sample Holder Calibration Wizard.**

12.17.1.5.2 Setting Calibration Options

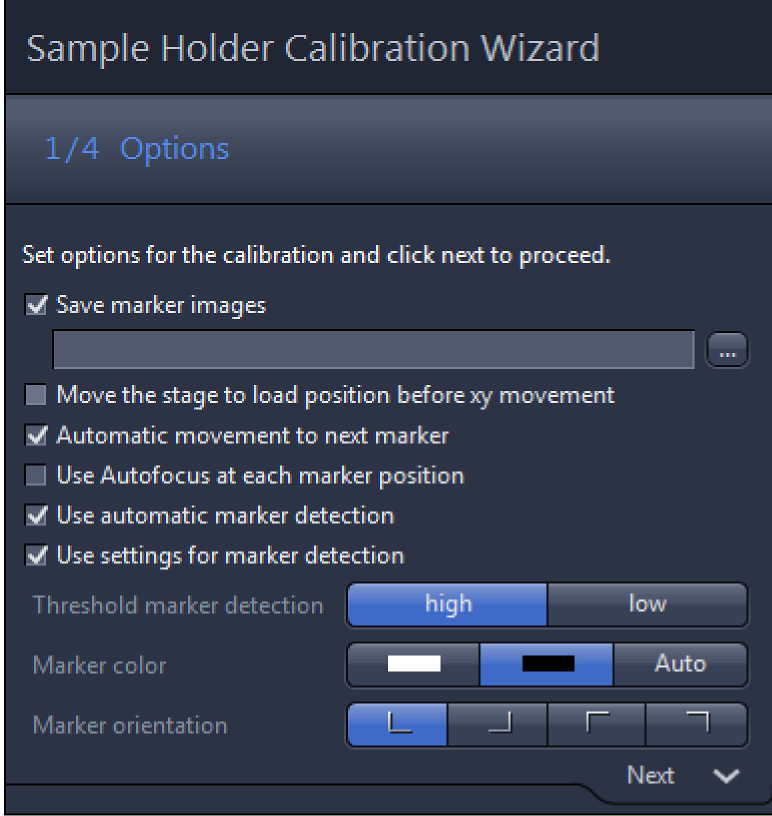


Fig. 32: Sample Holder Calibration Wizard Options

In step 1 of the wizard, the following options should be activated to follow our recommended workflow:

1. Check if the **Automatic movement to next marker** checkbox is activated.
   → This will automatically move the stage to the next marker position after you have confirmed the position of the marker and clicked on **Next**.
2. Check if the **Use automatic marker detection** checkbox is activated.
   → The software will try to find the correct positions of each marker automatically.
3. If you need to change the marker color, or check if the marker orientation is set correctly, activate the **Use settings for marker detection** checkbox to access these functions.
4. Click on **Next** to move to the next wizard step.

12.17.1.5.3 Performing Calibration

1. Click on **Set** to detect the first marker position.
   → An automatic stage calibration will be performed. After the stage calibration, the system will try to detect the marker position of the small marker automatically.
   → A message appears which asks if the marker was detected correctly.
2. Click on **Yes** to confirm the message.
3. Click on **Next** to move to the next step of the wizard.
   → The stage will automatically move to the next (coarse) marker position. If the stage moves into the wrong direction you can use the **invert X** / **invert Y** buttons to correct the movement direction.
4. Repeat the previous steps and set marker position 2 and 3 accordingly.

→ After setting marker position 3 you will find a green check mark icon which shows that the calibration was successful.

5. Click on **Finish** to save the calibration and close the wizard.

To check if the calibration was successful acquire an image and open the **Tree** view in the Center Screen Area. There you should see the correlative calibration data in the list. If the Tree view is not visible go to **Tools** | **Options** | **Documents** and activate the **Enable Tree View** checkbox.

12.17.1.6 Acquiring the LM Image

Basically image acquisition is performed as you are used to do it within ZEN software. The file format for Shuttle & Find data is the common ***.czi** file format. Saved images can be loaded in ZEN via the menu **File** | **Open**.

After image acquisition the next step in the correlative workflow is to define / draw in ROIs / POIs in your image. Therefore you can use the **Region** tools on the **S&F** tab, see *Regions, Find and Dimensions [▸ 490]*.

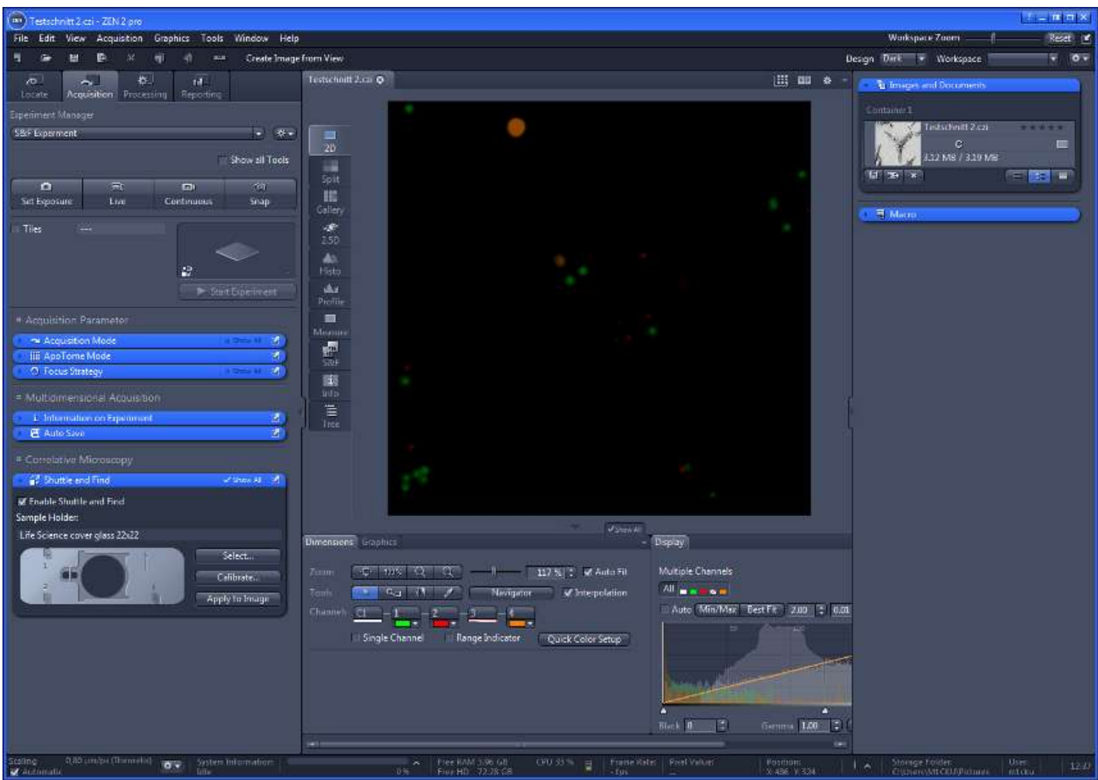


Fig. 33: LM image

12.17.2 Shuttle & Find Sample Positions at the Electron Microscope

Now you can transfer (Shuttle) the sample and the LM (Light Microscope) image file (**.czi**) to the SEM (Scanning Electron Microscope). There you can easily relocate (Find) the same sample positions and acquire a corresponding image within the ZEN **SEM** software. Therefore exactly the same steps have to be done as for the light microscope.

12.17.2.1 Mounting the Sample Holder to the SEM

For imaging your sample in the SEM, insert the sample holder **(2)** in the special SEM adapter **(1)** and mount it to the SEM.

Info

The arrow of the sample holder has to face the arrow of the SEM adapter.

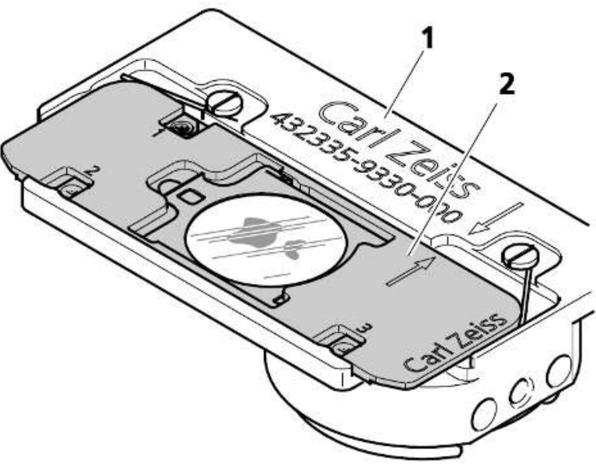


Fig. 34: Sample holder mounted in SEM adapter

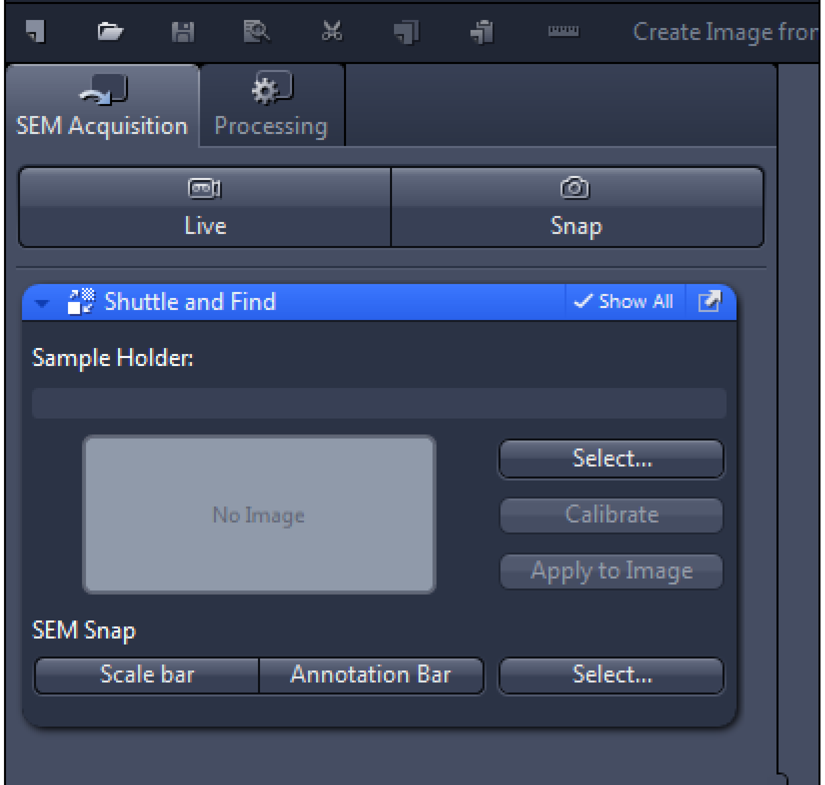
12.17.2.2 Starting the ZEN SEM Software

For correlative microscopy with scanning electron microscopes **SmartSEM** and ZEN (blue edition) SEM have to be installed. SmartSEM is still the control software of the scanning electron microscope. ZEN (blue edition) **SEM** comes as an add-on for SmartSEM to perform correlative microscopy and using Shuttle & Find on a SEM.

Prerequisite ✓ You have started SmartSEM.

1. Start the ZEN software by clicking on the program icon on your desktop.
   → The application selection window appears.
2. Click on the **SEM** button to start.

→ You will see the program interface with a reduced user interface comparing to the software. In the Left Tool Area the **SEM Acquisition** tab and the **Processing** tab are available only. On the SEM Acquisition tab you will find the **Shuttle & Find** tool which has 3 additional buttons at the lower part of the tool.



12.17.2.3 Selecting the Sample Holder

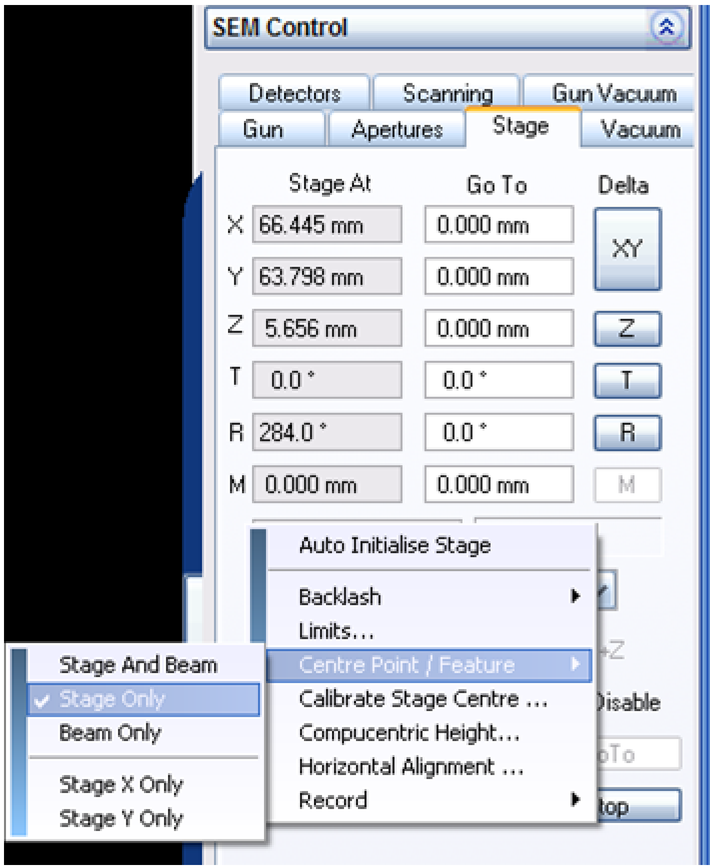
This step is exactly the same step like for the light microscope, so please read the chapter *Selecting the Sample Holder [▸ 474]* if you want to know the exact steps which you have to perform.

12.17.2.4 Calibrating the Sample Holder

Like the step before this step is exactly the same like for the light microscopy, so please refer to the chapter *Calibrating the sample holder [▸ 476]* for details.

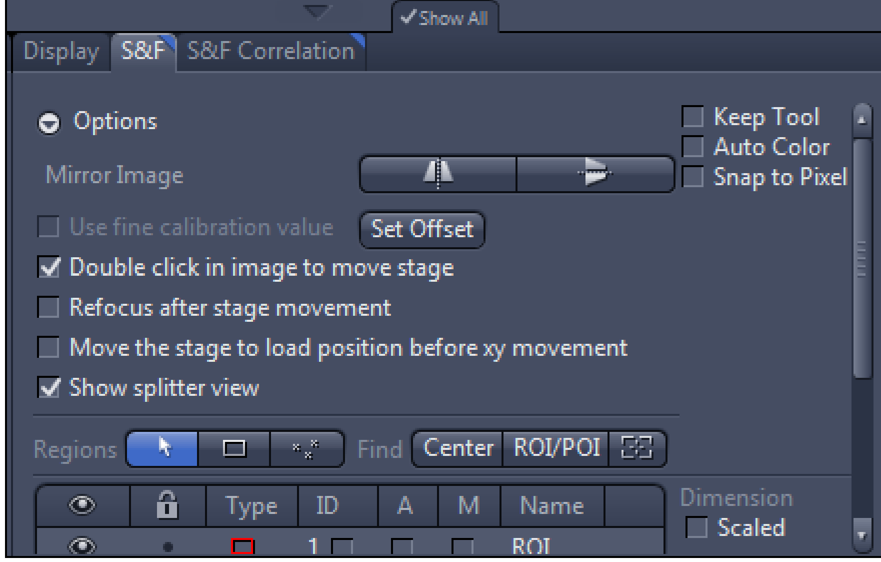
Info

- The calibration of the sample holder has to be done on both systems the LM and the SEM. Otherwise the relocation of your sample positions or ROIs / POIs stored in the image won`t be successful.
- Note that for Shuttle & Find the beam shift must be switched off. The beam shift is deactivated in **SmartSEM** as follows:
  - Call up the shortcut menu **Center Point / Feature** by right-clicking on the **Stage** property page.
  - Select **Center Point / Feature** and select **Stage only**.



12.17.2.5 Acquiring an EM Image

1. Load your LM image to ZEN SEM (.**czi**).
   → The image will be displayed in the center screen area.
2. Activate the **Live** mode.
   → You will see the Live image from the SEM. Notice that all settings for the SEM image have to be done within the SmartSEM software.
3. Activate the **S&F View** in Center Screen Area.
4. Go to the **S&F** tab.
5. Check if the **Double click in image to move stage** and **Show splitter view** checkboxes are activated (default setting).



→ In the left image container you see the live image from the SEM. The right image container is empty.

6. Drag the loaded LM image from the **Images and Documents** gallery into the empty image container.

Now you can easily relocate sample positions by double clicking within the image or on the ROI/POI button (if ROI / POI are drawn in and selected) on S&F tab.

For image acquisition you have to use the **Snap** button within ZEN SEM. Notice that we will not describe setup and image acquisition with the SEM. Please read the online help or user guide for the SEM software.

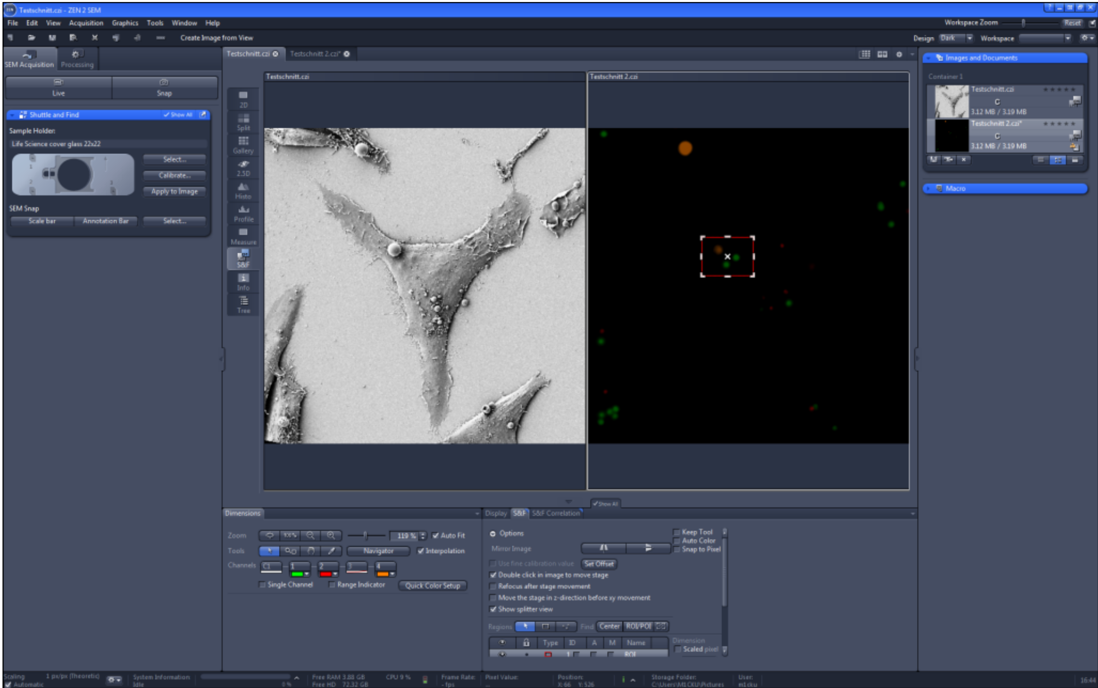


Fig. 35: SEM and LM image

12.17.2.6 Fine Calibration of the Sample Holder

The precision of relocation can be improved by determination of an offset value. This value describes the position offset between the loaded image and the live image. The defined offset value is only valid for the loaded image. If another image is loaded or if you close the dialogue, the offset value will be deleted.

Prerequisite ✓ An offset is visible when you try to relocate marker positions on the live image comparing to the LM image.

1. Click on the **Set Offset** button.
   → The stage moves to the selected marker position. Then a message appears which asks you to move the stage to the correct position.
2. Move the stage manually to the correct position by using the joystick.
3. Confirm the message by clicking on the **OK** button.

Now you can repeat the relocation. The positions should be identical now.

12.17.3 Correlating Two Loaded Images

Prerequisite

- ✓ You have acquired and loaded two images containing S&F calibration data (e.g. LM / SEM) to be correlated. If the images are not oriented identically you can use the **Mirror Image** buttons under **Options** on the **S&F Correlation** tab.
- ✓ You see the two images next to each other (splitter view) in the center screen area. If not, drag your images from the **Images and Documents** gallery into the center screen area.

1. Click on the **Set correlation points** button in the **S&F Correlation** tab.
   → The cursor will change to a pipette symbol.
2. Click in the left image to set a correlation point. Set all 3 marker points in the left image first, before you set the corresponding 3 markers in the right image. If a correlation point is set, a check mark icon will appear in front of the corresponding point.
   → Make sure that the positions in both images are identical. After you have set all 6 points the cursor will be changed backwards from the pipette to the arrow.
3. Click on the **Create Correlation** button.

The correlated image will be generated and opened in a new image container.

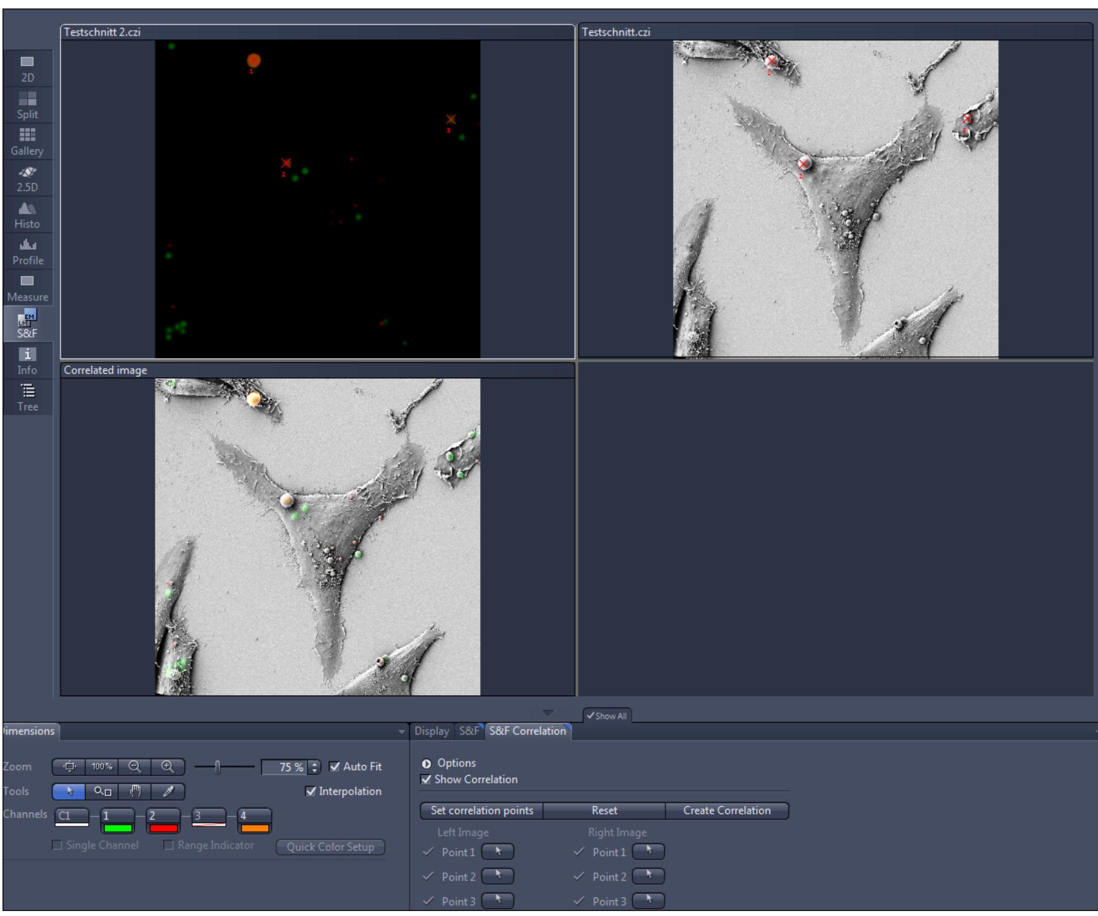


Fig. 36: Correlated image

Tips & Tricks

- It is also possible to set each correlation point individually. Therefore under **Left Image** / **Right Image** click on the **Arrow** button behind a point (e.g. **Point 1**). Then click on the desired position within the image.
- To improve the accuracy of the identification you can zoom into the images by using the mouse wheel.

- To edit/move a point, click on the point you would like to move. When the point is marked with a dashed rectangle you are able to move the point by holding the left mouse button. Alternatively, below **Left Image** / **Right Image** click on the points **Arrow** button you want to move and click on a new position within the image.

12.17.4 Correlation of Live Image and Loaded Image

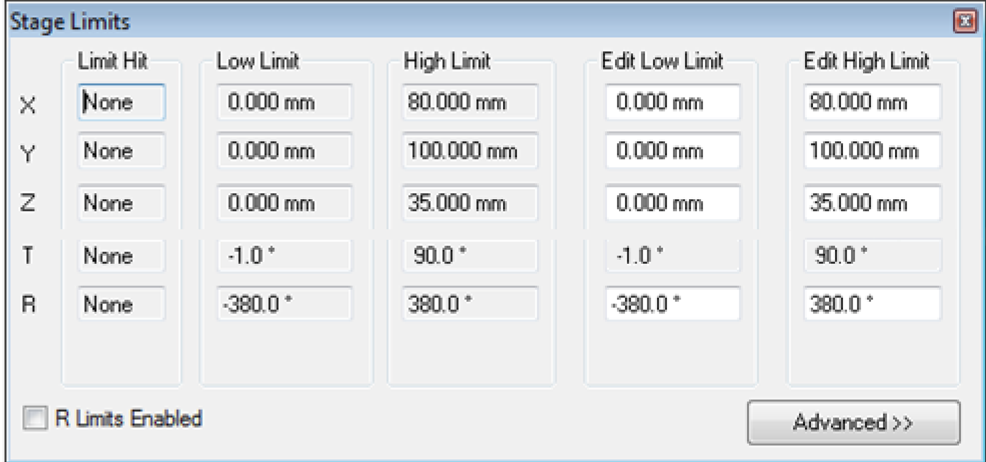
Prerequisite ✓ You have activated the **Live** mode.

1. Select the **S&F** view in the **Center Screen Area** and click on the **S&F Correlation** tab.
   → The splitter view will become visible in the Center Screen Area. In the left image container you see the live image.
2. Drag the corresponding LM image from the **Documents and Images Gallery** into the Center Screen Area.
3. Click on **Set correlation points** button to set the correlation points. Always start with setting 3 points in the left (live) image, then continue with setting the identical points (in the same order) in the loaded image.
4. After setting all 6 correlation points the image correlation will be performed automatically.

The correlated image will be visible in a third image container below the live image and the loaded image.

12.17.5 Shuttle & Find with an EVO 10

To use Shuttle & Find (SW and correlative holders) with an EVO 10 make sure that the stage limits (for x, y and z) are set as follows:



Holder Positions

The holder positions must be oriented like shown in the images

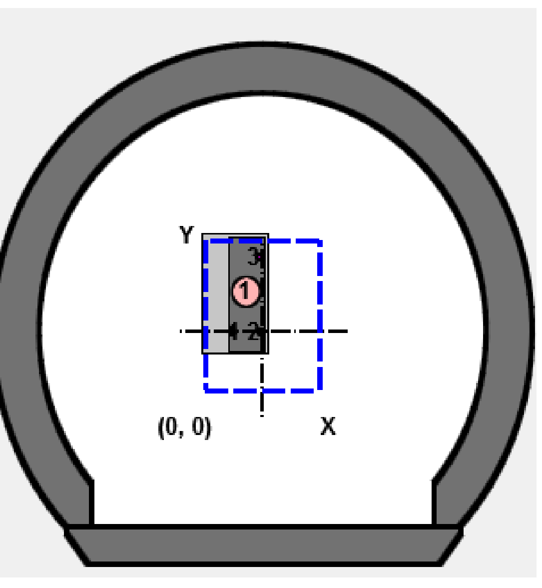
Position 1 Position 2 Position 3

NOTICE

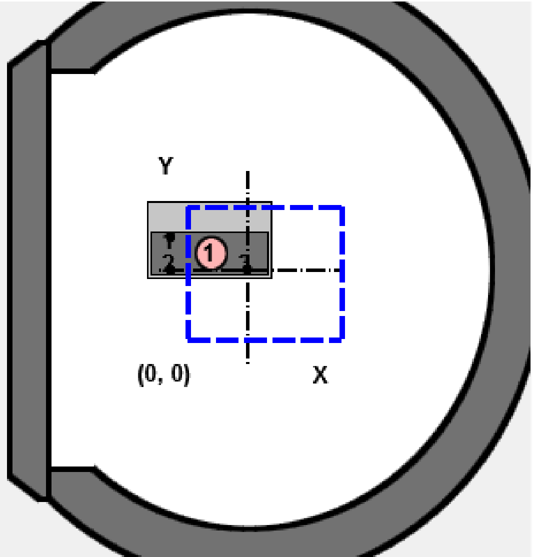
If you set a wrong orientation the stage cannot be moved to all correlative markers because of the stage limits for the EVO 10.

- The holder has to be mounted into the EVO in that the way that the correlative markers (**1**) and (**2**) have to be near the chamber door whereas marker (**3**) is located furthest from the chamber door (see **Mounting A/B**).
- If necessary, the SEM image can be rotated according to the LM image using the option **Scan Rotate** in SmartSEM.

Mounting A:



Mounting B:



12.17.6 Functions & Reference

12.17.6.1 User Interface

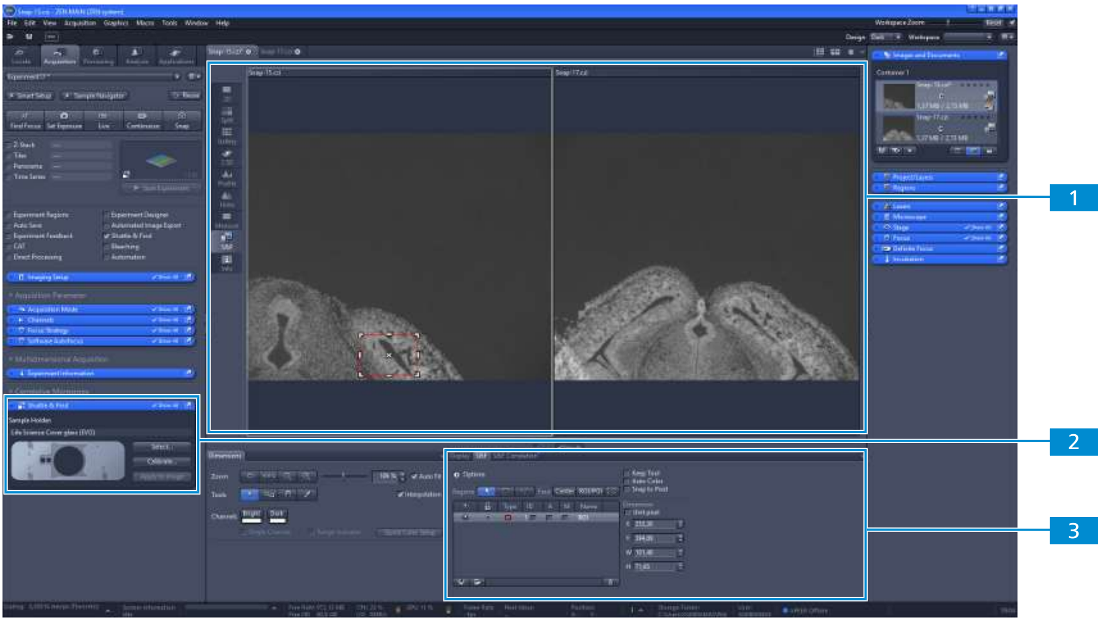


Fig. 37: User Interface

1 **Shuttle & Find View**
Area where the images are displayed. For more information, see *Shuttle & Find View [▸ 489]*.

2 **Shuttle & Find Tool**
Here you choose and calibrate your sample holders. For more information, see *Shuttle & Find Tool [▸ 487]*.

3 **Shuttle & Find View Options**
Here the **Shuttle & Find** specific view options are available. For more information, see *S&F Tab [▸ 489]* and *S&F Correlation Tab [▸ 492]*.

See also

Sample Holder Calibration Wizard [▸ 493]

12.17.6.2 Shuttle & Find Tool

Here you choose and calibrate your sample holders. The tool is visible only if you have activated the **Shuttle & Find** checkbox in the **Experiment Manager**.

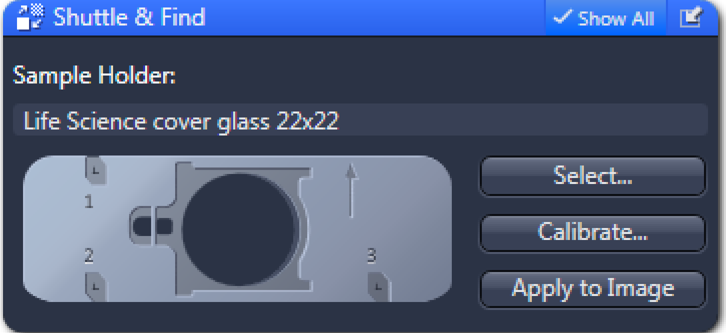


Fig. 38: Shuttle & Find Tool

Parameter	Description
Sample holder	Here you see the name and preview of the selected sample holder.
Select...	Opens the **Select Template** dialog. There you select the preferred sample holder or define new holder templates, see *Selecting the Sample Holder [▶ 474]*.
Calibrate...	Opens the *Sample Holder Calibration Wizard [▶ 493]*. There you can calibrate the selected sample holder.
Apply to Image	Only visible if the **Show All** mode is activated. **Use this button only, when you forgot to calibrate the holder before you acquire the image.** Applies a calibration to an acquired image. Do not remove the sample out of the correlative holder between image acquisition and calibration.

Shuttle & Find tool for SEM

Only visible if you have started the ZEN SEM software.

The tool window is adapted to the requirements of the correlative workflow on a SEM. Therefore three additional buttons are available.

Fig. 39: Shuttle & Find tool in ZEN SEM software

Parameter	Function
Scale bar	Adds a scale bar to the snapped (acquired) image.
Annotation bar	Adds an annotation bar to the snapped (acquired) image.
Select...	By clicking on this button a dialog opens to select parameters for the annotation bar. You can select max. 9 parameters for the annotation bar.

12.17.6.3 Shuttle & Find View

Besides the **Shuttle & Find** tool in the **Left Tool Area**, the **S&F (Shuttle & Find)** view is visible in the **Center Screen Area** of the ZEN software. If the S&F view is selected, the **S&F** tab and **S&F Correlation** tab will appear as specific view options under the image area.

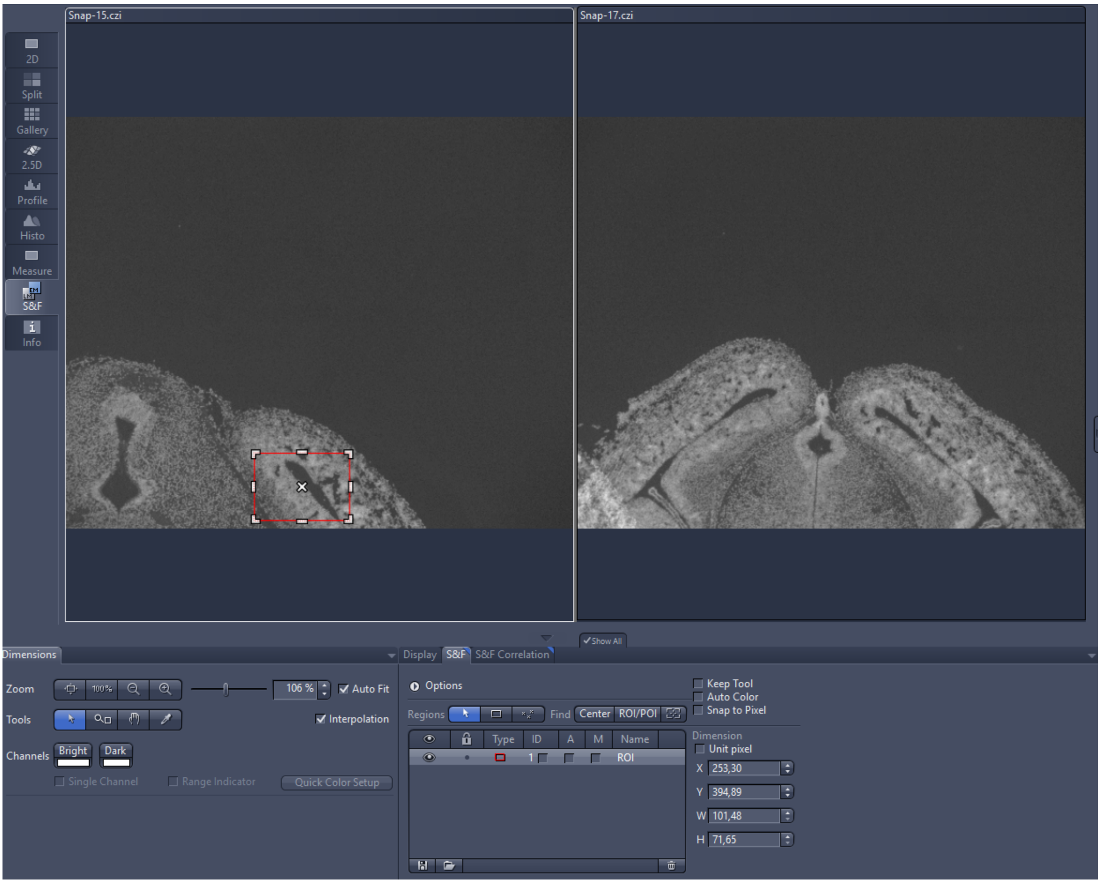


Fig. 40: Shuttle & Find View

12.17.6.3.1 S&F Tab

Here you find helpful options and tools to draw in and relocate regions of interests (ROIs) or points of interest (POIs) within the sample image.

12.17.6.3.1.1 Options

Parameter	Description
Mirror Image	Here you can mirror the image **horizontally** or **vertically** by using the two buttons at the right. The alignment of the images depends on the microscope (upright/inverted) and orientation of the sample holder.
Keep tool	**Activated:** Keeps the current tool active. That's helpful if you want to draw in more than one ROI/POI.
Auto color	**Activated:** Uses a new color for each new element which is drawn in.
Snap to Pixel	**Activated:** Draws in graphical elements using the pixel grid.

Parameter	Description
Use fine calibration value	**Activated:** Uses the measured fine calibration. The precision of relocation and therefore the quality of the overlay image can be improved by determination of an offset value. This value describes the offset between the loaded image and the live image. The defined offset value is only valid for the loaded image which you can see in the container. If another image is loaded or if you close the dialogue, the offset value will be deleted. Determine the offset by identification of a POI (Point Of Interest) within the snapped image. To identify a POI use the buttons in the **Regions** section. By clicking on the **Set Offset** button, the stage moves to the supposed sample position. Compare the sample position within the live image with the set POI and correct the stage in that way that both shown positions are identically. Confirm the fine calibration with the **OK** button. Now the fine calibration is measured and the checkbox is activated. More information, see *Fine Calibration of the Sample Holder [▸ 483]*.
Double click in image to move stage	**Activated:** Moves the stage to the position you have double clicked on.
Refocus after stage movement	**Activated:** Adjusts the focus automatically after the stage has moved.
Move stage in z-direction before x/y movement	**Activated:** Moves the stage to the load position before it moves to the next correlative calibration marker.
Show splitter view	**Activated:** Activates **Splitter** Mode in the **Center Screen Area**.

12.17.6.3.1.2 Regions, Find and Dimensions

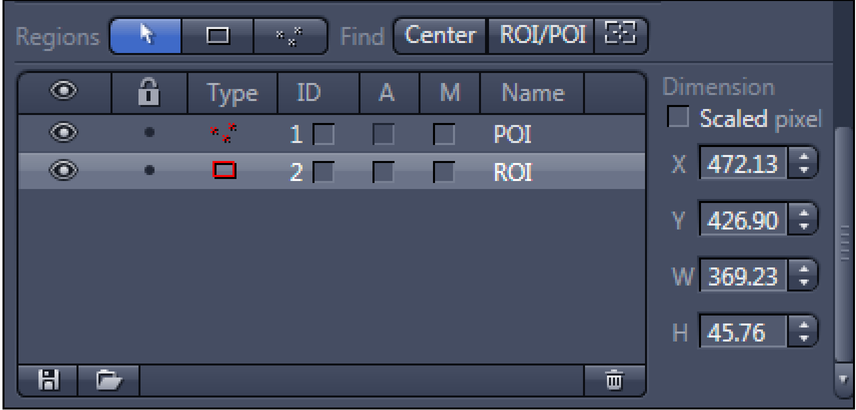


Fig. 41: Regions, Find, Dimension

Regions and Find tool bar

Parameter	Description
Selection mode	Selects the ROIs or POIs in the image area. If you are currently in another mode, you can switch back to the **Selection** mode using this button.
Draw rectangle	Draws in a rectangle (**Region of Interest (ROI)**) that is always parallel to the edges of the image.
Draw marker	Draws in a marker point (**Point of Interest (POI)**).
Center **Center**	Moves the stage to the center of the opened image.
ROI/POI **ROI / POI**	Moves the stage to the selected **ROI** / **POI**.
Show stage position	Shows the current stage position as a rectangle in the image.

Dimension section

Here you see coordinates and dimensions of the selected graphical element in the list. If the **Scaled** checkbox is activated, the unit is µm, otherwise Pixel.

- Parameter **X**: Shows the horizontal position (x coordinate) of the center of the graphical element.
- Parameter **Y**: Shows the vertical position (v coordinate) of the center of the graphical element.
- Parameter **W**: Shows the width of the graphical element.
- Parameter **H**: Shows the height of the graphical element.

Graphical elements list

Here you see the list of all ROI / POI which are drawn in.

Parameter	Description
Eye symbol	Shows or hides the ROI / POI in the image.
Lock symbol	Locks a ROI / POI to prevent changes.
Type	Displays the icon for the tool type (ROI/POI). To format a graphic element, double-click on the icon. The **Format Graphic Elements** dialog opens.
ID	Only visible if the **Show All** mode is activated. Displays the ID for the graphic element. To do this, activate the checkbox at the corresponding list entry.

Parameter	Description
A	Only visible if the **Show All** mode is activated. Displays annotations for a graphic element (ROI). To do this, activate the checkbox at the corresponding list entry. Then double click on the checkbox. The **Format Graphic Elements** dialog opens. Choose an annotation you want to have displayed within the image from the **Annotation** dropdown list.
M	Only visible if the **Show All** mode is activated. Displays measurement data for a graphic element. To do this, activate the checkbox at the corresponding list entry.
Name	Displays the name of the graphic element. To change the name, double-click in the Name field. Then enter the text of your choice.

12.17.6.3.2 S&F Correlation Tab

Here you find all functions to overlay (correlate) two images.

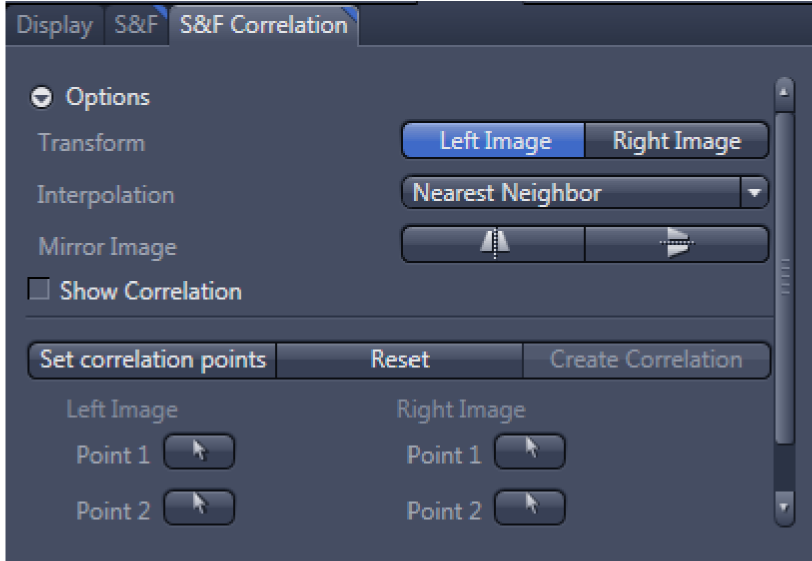


Fig. 42: S&F Correlation tab

Parameter	Description
Transform	Here you select which image will be transformed. Choose via the **Left Image**/**Right Image** buttons, which image should be transformed in the other. During transformation a pixel in the overlay image is calculated by using pixels of the two original images that shall be overlaid / merged.
Interpolation	Here you can select one of the following interpolation methods:
- Nearest Neighbor	The gray value of the resulting pixel in the overlay image is made of a pixel which is located next. This interpolation method is very fast.
- Linear	The resulting or calculated pixel in the overlay image is assigned to a gray value, which is the result of a linear combination of gray values derive from pixels located nearby (in the original image).

Parameter	Description
- Cubic	The calculated pixel in the overlay image is assigned to a gray value, which is calculated by means of a polynomial function using gray values of pixels in the original images; these pixels are located nearby the calculated pixel.
Mirror image	Here you can mirror the image horizontally or vertically. Therefore simply click on the corresponding button. Mirroring an image is necessary, when the loaded image shows a different orientation than the live image.
Show Correlation	**Activated:** Opens the correlated image in a new image document / new container.
Set correlation points	Enables you to set 6 points (3 points in each image) as correlation markers in a row, see *Correlating Two Loaded Images [▸ 484]*.
Reset	Deletes all correlation points in the images.
Create Correlation	Active only, if all correlation points are set in both images. Creates a correlative overlay image. A third image container with the correlated image will be opened in the **Center Screen Area** and the **Show Correlation** checkbox will be activated automatically.

12.17.6.4 Sample Holder Calibration Wizard

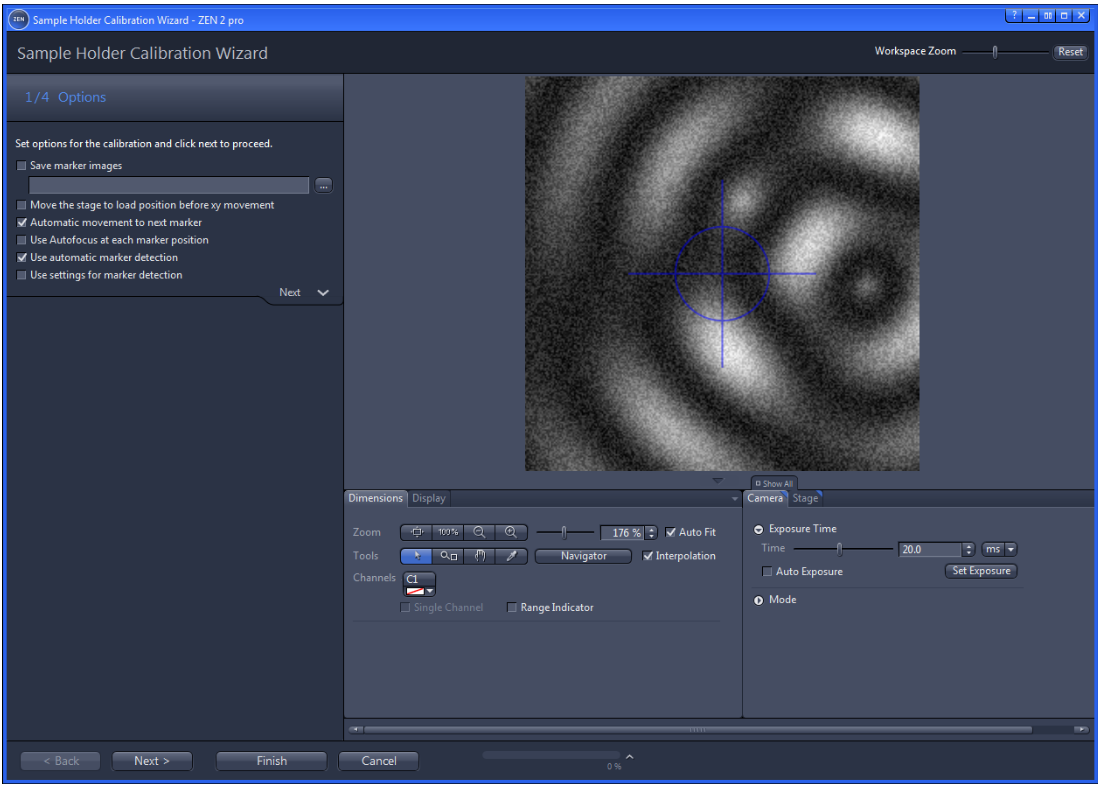


Fig. 43: Sample Holder Calibration Wizard

With the Sample Holder Calibration Wizard you calibrate your selected correlative sample holder. The wizard is opened via the **Shuttle and Find** tool. Make sure that you have activated the Shuttle and Find tool and selected a sample holder, see *Selecting the Sample Holder [▸ 474]*.

12.17.6.4.1 Step 1: Options

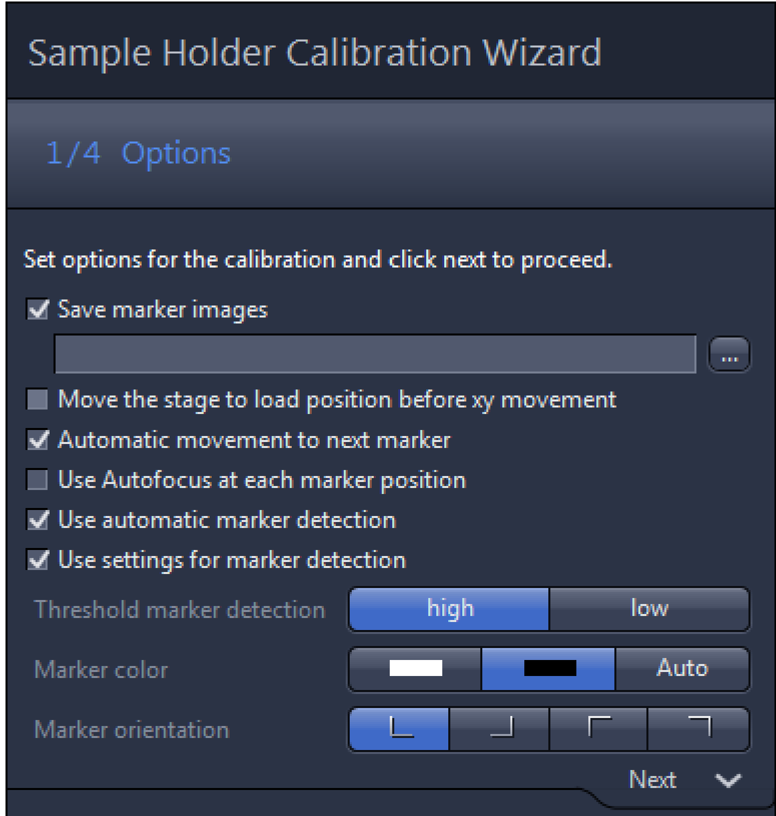


Fig. 44: Sample Holder Calibration Wizard Options

Option	Description
Save marker images	**Activated:** the marker images are saved during the calibration. The images can be used to check the calibration afterwards. Click on the **Select Folder (...)** button to select a storage folder.
Move the stage to load position before x/y movement	**Activated:** the stage will move to load position before moving to the next correlative calibration marker. In case of using an AxioObserver, the objective revolver moves to load position.
Automatic movement to next marker	**Activated:** By clicking on the **Next** button within the wizard the stage moves automatically to the next calibration marker.
Use Autofocus at each marker position	This option is active only if the **Automatic movement to next marker position** checkbox is activated. **Activated:** the focus is adjusted automatically after moving to the next marker position.
Use automatic marker detection	**Activated:** The software will try to detect the small calibration marker automatically.
Use settings for marker detection	This option is active only if the **Use automatic marker detection** checkbox is activated. **Activated:** shows settings for marker detection (see description below). Here you select the properties of the calibration markers.

Settings for marker detection

Only visible if the **Use settings for marker detection** checkbox is activated.

Option	Description
Threshold marker detection: high – low	A low threshold for marker detection is used when the dimensions of the correlative L markers cannot be recognized precisely, e.g. when the sample holder is slightly filthy.
Marker color	Here you select the color of the markers displayed in the live image. **White:** the marker is displayed white on a dark background. **Black:** the marker is displayed dark on light background. **Auto:** the marker color is set automatically.
Marker orientation	Here you need to set the orientation of the L-markers on your sample holder. Click on the corresponding button to select the orientation of the calibration marker which you can see in the live image

If you click on the **Next** button you will move to the next step of the wizard.

12.17.6.4.2 Step 2-4: Calibration

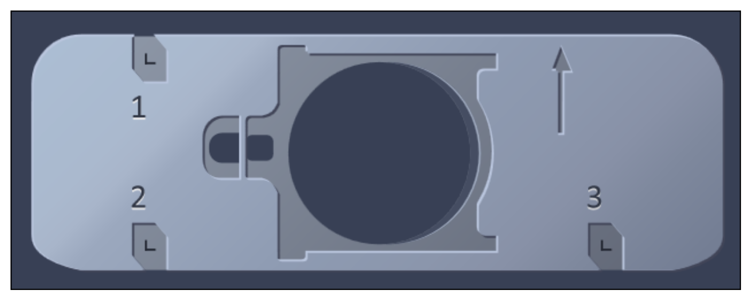
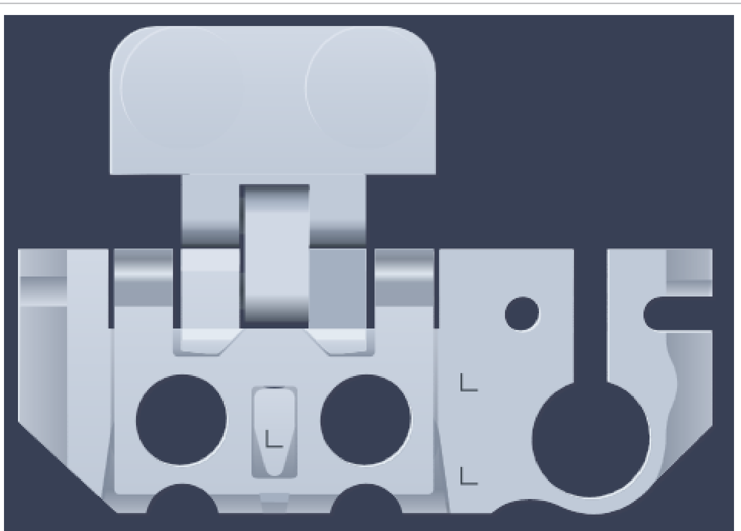
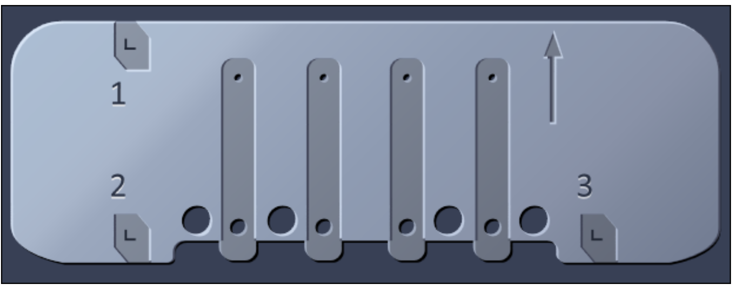
In steps 2-4 of the wizard you will be guided through the calibration procedure.

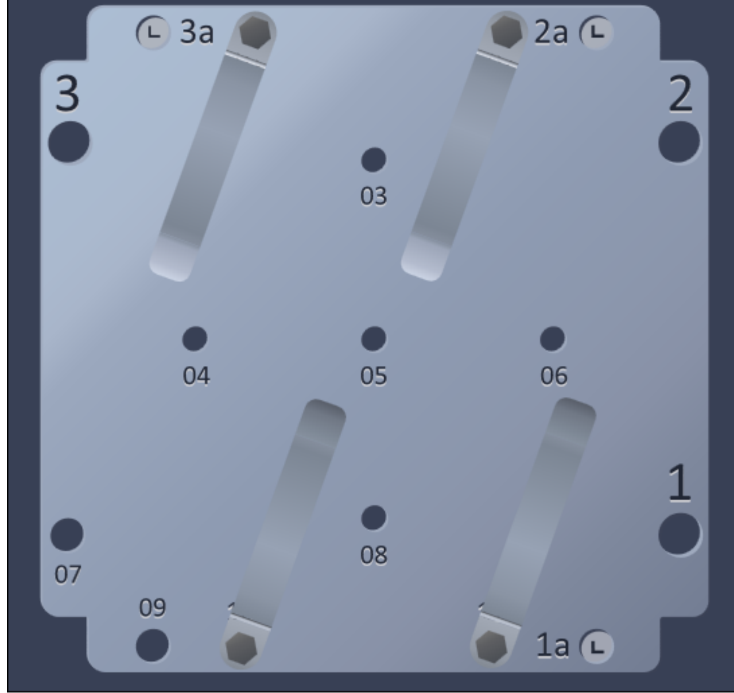
Fig. 45: Sample Holder Calibration Wizard

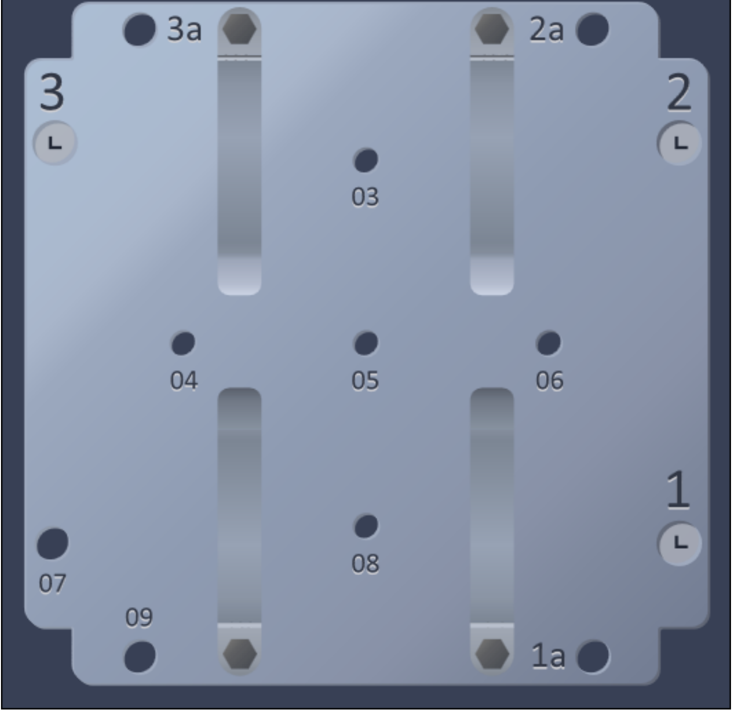
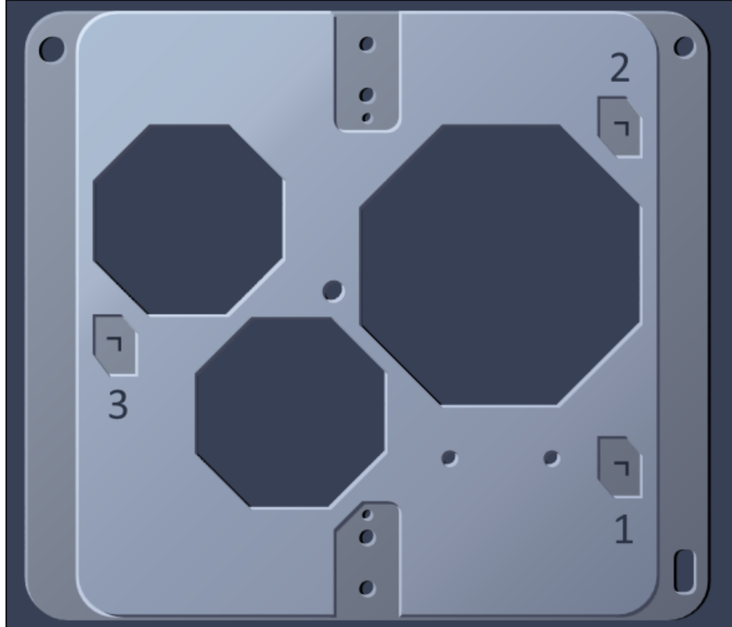
Option	Function
Holder position	**Move to Position 1** button Moves the stage to marker position 1. This is possible only if the first position was set before and x/y coordinates are given. **Current** button Only visible for marker position 2 and 3.

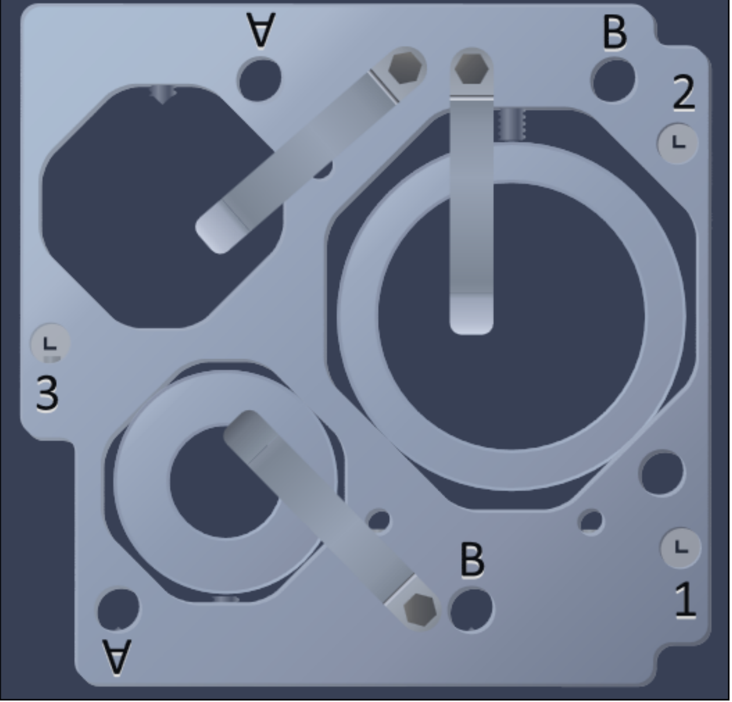
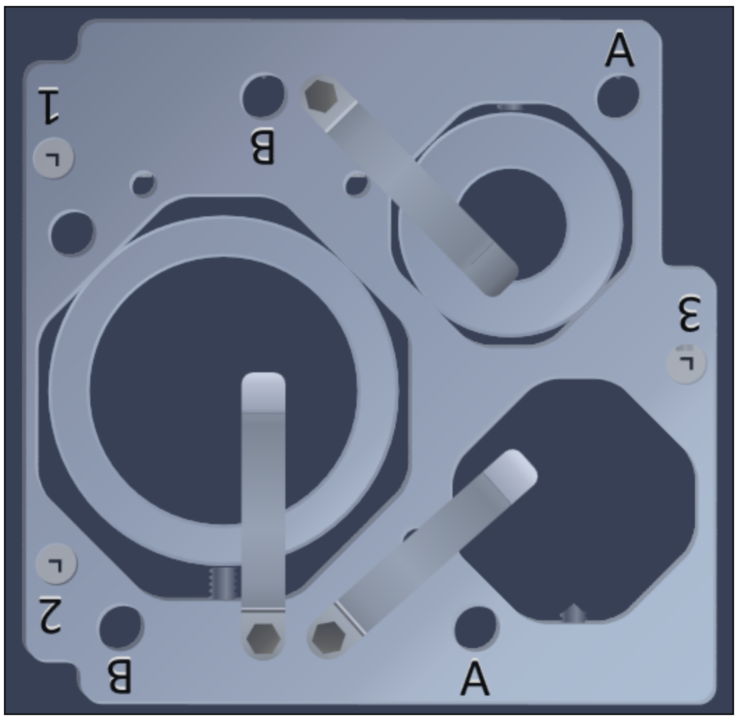
Option	Function
	Moves the stage to the current marker position. This is possible only if the current position was set before and x/y coordinates are given.
Stage movement to the next marker	Here you can change the movement of the stage in x or y direction. This is necessary if during calibration the stage moves in the wrong direction.
Marker position	By clicking on the **Set** button, the actual marker position will be confirmed.

12.17.6.5 Correlative Sample Holders

Name	Image
Life Science cover glass 22x22	
Life Science Cryo Holder	
Life Science for TEM Grids	

Name	Image
Cover glass with fiducials 22 x 22	
MAT Flat Stubs A	

Name	Image
MAT Flat Stubs	
MAT Universal A	

Name	Image
MAT Universal B_A	
MAT Universal B_B	

12.18 Software Autofocus

This module offers a configurable image based autofocus functionality that will search through a series of axially stepped images analyzing the “sharpness” of each. The z-value of the image returning the maximum sharpness is set as the new plane of observation.

The module requires the microscope to be fitted with a motorized Z-drive. It does not require a z-piezo actuator nor is the z-piezo used by the software autofocus (SWAF) in the current implementation. On the **Acquisition** or **Locate** tab the settings for the SWAF can be adjusted in the **Software Autofocus** tool. These can also be called (and tested) by clicking the Find Focus (AF) button on the main button bar on Acquisition tab. SWAF settings are stored as part of an experiment on Acquisition tab.

The configuration allows the function of the SWAF run to be matched to the conditions under which the focus should be found. A basic description of the functions adjusted by the individual controls can be found in the chapter Functions & Reference. However, before going into the description of each parameter, we will try to address the following questions: How does the SWAF in the software attempt to locate the “focus”? And how do the parameters settings influence its behavior in this respect?

12.18.1 Terminology & Abbreviations

Perhaps the best place to start is with an explanation of the terms encountered when working with focus strategies before looking at the individual strategies in detail. Many of these terms are also encountered in the **Tiles** and **Software Autofocus** module. The nomenclature takes some time familiarize with due to its subtleties. Here is a list of the more common terms:

Term / Abbreviation	Description
SWAF	Stands for Software Autofocus.
DF,**DF.1** or **DF.2**	Stands for Definite Focus, Definite Focus.1 or Definite Focus.2
Tile	One of the individual image fields that make up a tile region i.e. a 2x2 Tile region is made up of 4 tiles arranged as a grid. The tiles have a given overlap with their neighbors (default setting 10 %) allowing them to be stitched together as one image if necessary. Unless otherwise specified by a focus strategy, each tile has the same z-value as the parent Tile region. After acquisition the individual tiles are displayed together as part of the tile region to which they belong, which in turn makes up one scene.
Tile Region	In a tile experiment a tile region refers to a ordered group of individual image fields (or tiles) that belong together and are arranged in the form of a grid (these arrangements can be based on quadrilaterals, circles, ellipses or freehand polygons) with a predefined overlap (default 10%) to facilitate stitching the images together. With the help of tile regions it is possible to acquire areas with dimensions that vastly exceed the size of an individual image field. Within an experiment a number of tile regions can be acquired at various localities/ wells/ containers on the sample. Each tile region is based on an X and Y coordinate of the stage and a Z coordinate of the focus drive and are defined using the Tiles tool. After acquisition the individual tile regions are displayed as scenes to facilitate viewing.

Term / Abbreviation	Description
Position	In a tile experiment positions refer to independent, individual image fields that placed at various locations on the sample. A position corresponds to (or is in some ways equivalent to) a tile region consisting of just one tile. Each position is based on an X and Y coordinate of the stage and a Z coordinate of the focus drive. Individual positions or position arrays (grouped individual positions) are defined using the Tiles tool. After acquisition the individual positions are displayed as scenes.
Reference Channel	The channel selected as a reference z-value for focus strategies and events in particular a SWAF. The selected reference channel can be changed in the Reference channel expander or in the Channels tool. It is also possible to define a relative axial offset to the reference channel. This can be done for one or more other channels.
Focus Surface	Refers to the interpolated surface of z-values derived from support points (discrete z-values) defined by the user (or by functions such as SWAF or DF.2) prior to the experiment (or immediately before acquisition start). A focus surface can be "local" or "global". The local form is confined to a single Tile region and attempts to describe the sample topography covered by the tile region such that all its image fields (tiles) will be in focus. The global surface form is technically identical, but is associated with a sample carrier, and defined in the sample carrier template dialogue. Thus, tile regions or positions placed on this carrier will follow the slope or contour defined by a topography that covers part or most of the sample carrier. In both cases the surface is defined by interpolation from discrete z-values – so called "support points". Note that a positions z-value is used as its local surface, and as such does not require a support point. Global and local surfaces cannot be mixed in a single experiment (or block).
Support Point	To create a focus surface it is necessary to define one or more support points. Support points are user defined collections of z-values that correspond to the desired plane of observation at a given XY-coordinate. They can also be defined initially by a SWAF run or DF.2 recall focus function- initially, after the experiment is started, but before the first loop of images are acquired. The number of support points, defined by the user, can be distributed automatically by an algorithm, re arranged individually by hand or placed at the current stage position. The number of support points employed determines the degree of interpolation that can be used to generate the topography of the focus surface. Typically, the interpolation criteria (minimum number of support points required to generate a certain degree) should be over filled with support points, and a lower interpolation degree selected for more robust results. By default the software employs an interpolation degree of level 2 (which can generate a parabolic saddle surface with at least 9 support points). If too few support points are used the next lower level (a "tilted plane") will be use automatically. Higher interpolation degrees have to be manually selected, but for most use cases are typically not necessary.

Term / Abbreviation	Description
Z-value	The current Z coordinate of the focus drive that is used to define a Tile region, position or support point when it is created by the user. Note that the individual tiles of a tile region all have the same initial z-value unless support points are used either in the context of a local or global focus surface, a software autofocus is used to determine them individually or a definite focus stabilization adjusts them. The z-value of a position defines its z-coordinate initially when a local focus surface is used. Positions spread on a global focus surface (carrier based) are adjusted accordingly as are the individual image fields of a tile region.
Adapt Z Values/ Focus Surface	The focus strategy **Use Z Values/ Focus Surface defined in Tiles Setup** allows the Focus surface or z-values defined in the Tiles tool to be modified by the result of a SWAF or DF stabilization based on these initial values. These functions are not available when no SWAF module is present or no DF is configured. The function has several module / hardware dependent variations:
- As Additional Action	In focus strategies that use a focus surface or z-value defined by the Tile setup (tool) it is possible to optionally execute a so called "additional action" (a stabilization event) that adapts the focus surface/ z-values. This occurs after the reference z-value has been reached as defined in the tiles set-up for each discreet z-value (i.e. each tile/position or the defining focus surface). Depending on the system configuration this can be a SWAF run or a DF stabilization. In the case of a SWAF run the initially defined reference z-value is used to center the search range defined in the SWAF settings. Thus, a SWAF run can be centered on the sample topology increasing the effectiveness and/ or speed at which a maximum is detected and subsequently used for image acquisition. In certain applications, such as Correlative array tomography (CAT) this function can be performed with DF instead. In this case a local focus surface is used to make sure that the DF stabilization stays within the catchment range of the device (only important for DF.1!). Complimentary to this is the number of support points needed to initially define the surface can be significantly reduced for a large elongated Tile region - which greatly reduces set-up time to image the extremely thin (typically 70 nm thick or less) "ribbon" of serial sections.
- Update with Single Offset	In combination with a Definite Focus or SWAF if a time series is used it is possible to make use of a focus surface or z-value defined by the Tiles setup and execute a so called "update" (a stabilization event) – this makes use of a SWAF run or a DF stabilization to update the Focus surface/ z-value defined initially by the Tiles set-up. In a time series the update action is performed once each time point (or every nth) at a single discrete "wait position" (default center of 1st Tile region / position). A change in Z (thermal or residual focal drift) at the wait position - if detected - is then applied to all the focus surfaces or Z-values defined in the Tiles Setup (adapting them all by the change in Z, applied as a common offset). In some cases it is useful to be able to define a specific waiting position – for example when a special sample carrier is used were the DF reflex signal might be disturbed by its structure/optical properties at the first tile region/position. Alternatively, if using a SWAF some kind of fiducial marker or such is available at this position that does not change (e.g. bleaching or movement) can be used.

Term / Abbreviation	Description
- Update with Multiple Offset	For Definite Focus.2 only an additional function is available that allows the device to be initialized on each and every z-value prior to the experiment and hence stabilize and update these individually according to their location relative to the sample/ glass interface. This function can be used with or without a time series dimension. Thus, DF.2 enables true multi-location experiments in which the user defined z-values (including support points) are used by DF.2 to create a stabilization map that is monitored and updated throughout the experiment.
Initial Definition for Z Values/ Focus Surface	This function allows you to select how the initial z-values used in the experiment are defined. By default this is **By Tiles Setup** and the z-values specified there (in the Tiles tool) are used. However, it is possible to define or adjust these z-values directly before the experiment (after clicking **Start Experiment**) either with a SWAF run or a with a DF.2 Recall Focus (Axio Observer). For the Celldiscoverer 7 this drop down offers the additional options **Find Surface** or **Find Surface + Additional Offset** to define the initial z-values. In this case the z-values are initially defined by the z-values resulting from this “pre-run” before the imaging loop starts. This can be particularly useful when working with multi well plates or chamber slides where the sample is located at a similar position relative to the carrier surface in each well or chamber. It also allows the imaging loop of the experiment itself to be speeded up and to be run in a triggered or compromised protocol (fast acquisition) thus reducing the time to complete the imaging loop of the experiment.
Stabilization Event Repetitions and Frequency	Defines the frequency and repetition of stabilization events within a given focus strategy. For the DF and SWAF focus strategies you can determine when and where in the experiment these event are executed in synchrony to the imaging loops – a loop here means time series, or positions for example, with the event synchronized to occur immediately prior to the chosen loop. A general limitation of this implementation (to limit code complexity) is that these stabilization events can only be synchronized to iterate with a single imaging loop entity i.e. the selection is only possible in a mutually exclusive manner. These settings can be accessed only when 'Show all' is activated and expert mode is selected. Initially default settings are assigned and can be restored by clicking the 'Standard' button. In 'Expert' mode the settings are displayed and can be, if necessary, modified. Depending on the dimensions of the experiment or focus strategy different parameters can be modified to meet the experiment needs. For the Tile Region loop you can optionally select were the event occurs within the Tile region - either in the center or at the 1st Tile of the region (typically upper left hand corner). This is of use when using SWAF events as often the upper left hand corner of a Tile region might not contain sample, thus often the SWAF run will not return a suitable maxima (new z-value). Finally, focus strategies that include Definite Focus and are used with a time series dimension may also allow stabilization during the interval of the time series i.e. asynchronous to the imaging loops of the experiment. This might be necessary if the time interval is on the order of tens of minutes, or if a large thermal drift is expected (more significant for DF.1), or if the time series has no or a very short interval (i.e. fast as possible acquisition at a single position) allowing synchronized events to be disabled completely.

Term / Abbreviation	Description
Focus Surface Outlier	Under **Tools > Options > Acquisition > Tiles** you find the option **Enable Removing of Focus Surface Outlier**. By two parameters you can define how so called "outlier" values are handled prior to calculation (interpolation) of a focus surface. This is particularly helpful when the z-values that will be used for this purpose contain one or more values that differ obviously from the others (for example if a SWAF run has returned a z-value that does not lie close the sample plane of interest). If not removed such values locally distort the focus surface potentially producing "blur" in the resulting images. By default a linear fit is used to detect such outliers in combination with a statistical threshold value (sigma). Values that do not meet these criteria (i.e. are significantly outside this) are classified as outliers and are not used to calculate the focus surface that will be subsequently generated for the experiment. In extreme use cases it is possible to modify the sigma value or use a mean value instead of a linear fit for this purpose, but typically these default values never need to be changed.

12.18.2 When is focus the "right" focus?

In microscopy, the focus can be implied from image parameters, such as the contrast or intensity, that vary with the position of the objective's plane of observation in the sample and the level of detail at a given plane. However, an algorithm that tries to detect (and maximize) such values will only return an axial position that corresponds to a plane of interest if these coincide (which is typically the case with (thin) samples with a singular discrete plane of detail).

This becomes increasingly difficult with higher numerical aperture (NA) lenses, thicker samples, and less pronounced levels of detail (modulated as change in contrast or intensity in the resulting image). Hence, SWAF is not to be understood as a focus finder, but can be used as a method for reliably searching over a given axial range and locating such a plane in a sample. Thus, although not all samples and imaging conditions will be appropriate, SWAF is an approach that allows a useful detection of a focus plane as a start for further imaging activities.

12.18.3 Software Autofocus in ZEN

Basically, the SWAF searches, with a pre-set z step size, within a given range of z values for the image plane that returns the maximal "sharpness" value. The step size or sampling rate of the SWAF is determined by the objective NA and wavelength (more details are given below). In turn the (automatic) search range is also largely determined by the objective NA – obviously optics dictate that higher NA objectives have smaller search ranges and vice versa. For SWAF to be useful for the application in question the image plane that returns the maximal sharpness should ideally be equivalent to the plane of interest in the sample – i.e. thus sample characteristics determine whether SWAF is the appropriate method to reliably detect the desired plane of observation. The component algorithms and functions of the SWAF, their relationships and the basic SWAF workflow are visualized schematically in the image below:

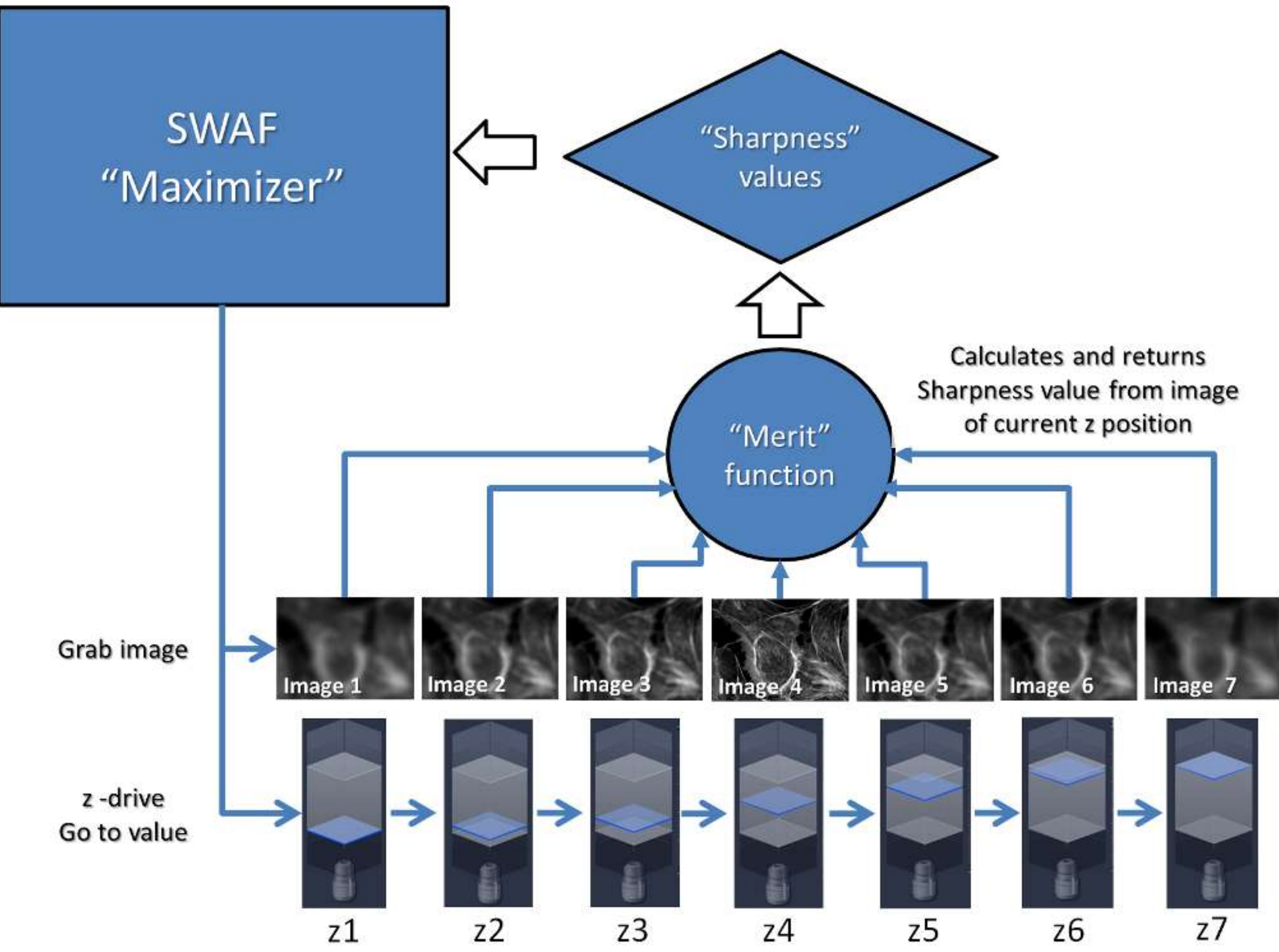


Fig. 46: SWAF Workflow

The SWAF run is driven and controlled by the Maximizer which in turn controls the z-drive and camera to acquire a sequence of images at predefined axial intervals. Each of these images is transferred in sequence to a merit function that calculates a sharpness value for the image. This value is returned to the maximizer were a table of sharpness values is maintained. The Maximizer searches for (using either unidirectional (“full”) or bidirectional (“smart”) axial travel) and determines when a maximal sharpness value is found. Next data fitting is performed locally around the returned maximum to refine its position. Subsequently, the SWAF sets this z-value as the resulting axial position of the z-drive. If no maximum is found the Maximizer detects an error condition and throws a corresponding exception (error message). In this case the z-drive returns to its position of origin, thus minimizing the likelihood of a sample/ objective collision incident.

12.18.4 FAQ

12.18.4.1 What should I do to adjust parameters of a SWAF run prior to using it in experiments?

In the first instance test the SWAF with the default settings and select an appropriate channel to be the reference channel – remember that you can uncheck the reference channel in the Channels tool i.e. so that it will not be imaged, but will still be used for a focus strategy or SWAF. Consider using a transmitted light channel if possible as this will not be subject to bleaching. Remember that the sample plane that returns a maxima from the SWAF run is not necessarily the same plane as that which you’re interested in imaging.

If this is the case, then you need to capture z-stacks with a suitable range or make use of the focus offset function that allows each channel to be offset by a relative value in z to the reference channel. Typically, once you have established that a maxima can be reliably found with the default SWAF parameters you might want to consider optimising the SWAF run by modifying the parameters to reduce the time required to complete it or to reduce sample exposure (phototoxic stress levels).

12.18.4.2 The SWAF returns an error message. What does this mean and how can I correct this?

The most typical error encountered is that the SWAF run does not find (return) a clear maxima and hence fails (hits so called search boundary). This might be because the step size set is too course (i.e. the maxima is missed occasionally) or the search range does not contain a clear sharpness maxima e.g. due to lower contrast/ intensity (signal to noise) or a lack of signal.

This can happen, for example, at the 1st tile (upper left) of a tile region which is empty such as is often that case in the corners of such tile regions. In this case it is possible to modify the SWAF focus strategy in the Tile Region loop such that the SWAF will be executed at the centre of the tile region. In ZEN 2.3 and higher if a SWAF failure occurs at a given loop entity then a fall-back applies such that the initial or last known z value will be used.

12.18.4.3 SWAF returns a failure after reaching a search boundary – what`s wrong?

If you are able to optimize the SWAF and the reference channel is typically robust for this then the solution might require that you look at the overall stability of the focus due to environmental variables such as vibrational isolation, temperature flux, evaporation of fluid from sample vessels, or poor lighting conditions (extraneous light).

Generally, if these are optimized then better overall results can be expected, if this is not the case then the fluctuations they cause might be the source of such reliability problems when detecting the sharpness maxima. Other things to consider are optical disturbances such as loss or poor immersion of the objective (bubbles), or artefacts caused by sample characteristics (for example (cell) debris moving across the FOV during the SWAF run). Perhaps it is possible to use a fiducial marker if the sample allows this (see Auto Focus ROI above).

12.18.4.4 When should I use the Full or Smart setting?

This depends entirely on the sample in question and manner in which it is imaged (e.g. reflected or transmitted light). However, if we are to generalize then the answer might simply be given as follows. A **Full** search will return the global sharpness maxima from the entire search range defined in the SWAF tool. The search always runs in one direction i.e. the z-movement of the actuator is unidirectional always moving against gravity.

Smart on the other hand is intended for quickly* detecting a local sharpness maxima by allowing a bidirectional search pattern. Thus, the full setting is typically a more extensive search that takes longer to accomplish. However, the smart setting may be suitable and saves a great deal of time and reduces sample light exposure.

*Typically, it will be the case that **Smart** is faster, but under some circumstances it might be slower. This will likely be the case when the maximum is far away from the starting position.

12.18.4.5 Can I change camera parameters in a SWAF run e.g. exposure time or binning?

In the implementation of SWAF in ZEN blue version 2.1 and earlier it was not possible to change the acquisition parameters directly. The exposure time setting using by the SWAF is based on the exposure time setting of the reference channel. Thus, longer exposure times of the reference channel increase the exposure time employed by the SWAF up to a maximum of ca. 100 ms. Thus, weak signals in the reference channel may cause an increased likely hood that no clear maximum will be found. Binning settings of a camera used by the reference channel are not taken into account and SWAF always uses 1x1 binning – this may cause issues with cameras processing smaller pixels (e.g .new AxioCam models).

In ZEN Blue version 2.3 and higher the SWAF has be further adapted to address the described limitations. Thus, exposure times defined for the Reference channel are used even when these exceed 100 ms. Settings that apply to all the channels defined in the acquisition mode tool still apply to the SWAF i.e. an independent binning setting for the reference channel is not yet possible. Thus, please take this into account when setting up your SWAF parameters. In addition, the SWAF run has been streamlined and can make use of triggered acquisition when supported by the camera making the SWAF run ca. a factor of two faster than in previous versions.

12.18.5 Functions & Reference

12.18.5.1 Software Autofocus Tool

Parameter	Description
Mode	Here you can select the sharpness measurement mode.
- Auto	This is the default setting. If selected, the software makes a choice based on the configuration of the microscope. Such that for Widefield approaches or transmitted light the sharpness measure is always **Contrast** based. On the other hand, for optical sectioning methods (e.g. Spinning Disk) the software will automatically select an **Intensity** based approach to determine the sharpness values. If the microscope configuration can`t be detected automatically you can manually select the sharpness measurement mode.
- Contrast	If selected the contrast based mode will be used for sharpness measurement. This is the standard setting for **Camera** acquisition.
- Intensity	If selected the intensity based mode will be used for sharpness measurement. This is the standard setting for **Confocal** acquisition.

Parameter	Description
- Reflex	Only available for **LSM** systems with imaging tracks other than MPLX and Airyscan SR. Using those tracks might lead to overexposure of the channel and failure for the Autofocus. If activated, the reflex of the laser on the cover glass surface is detected. An offset is used to focus onto the sample. You need to add the offset once by clicking the **Find Offset** button while the sample is in focus. The method requires a refractive index mismatch between the immersion medium and the cover glass and is therefore not for oil immersion objectives. For **Reflex** mode, the **Search** parameter should ideally be set to **Smart**. After the offset was defined, you can manually reduce the search range in order to facilitate shorter focusing times. The acquisition settings (e.g. laser line, PMT gain, emission filters) are configured automatically.
Quality	This parameter determines the merit function that will be used to calculate the contrast value of the image when **Contrast** mode is used by the SWAF (Software Autofocus) run to measure sharpness.
- Default	If selected, a composite of weighted merit functions is used. Use this setting if the sample cover a greater part of the camera field of view.
- Low Signal	If selected, a single merit function to determine the value is used. Use this setting if the image is noisy or the sample covers a small area of the field of view. As might be the case if you work with a calibration slide or beads.
Search	There are two options Smart and Full. These define a different type of primary maximizer used to run the SWAF, which in turn determines a number of additional characteristics and parameters of the entire process. To learn more about this, read the *FAQ entry [▸ 506]*.
- Smart	If selected, an alternative maximizer is used that can search in a bidirectional manner and will stop when a local maximum is found in the sharpness values (i.e. a significant decrease of sharpness in both z directions). Again if an error condition is detected the Smart maximizer will throw an exception. For specific information on the Software Autofocus using LSM Tracks in this context, also refer to chapter *Software Autofocus using LSM Tracks [▸ 510]*.
- Full	If selected, the maximizer employed with this setting uses a unidirectional movement of the z-drive stepping through the entire relative or fixed search range defined in the SWAF tool (see Autofocus Search Range). The Full maximizer will return a global maximum for the autofocus run or throw an exception when an error condition is detected. For specific information on the Software Autofocus using LSM Tracks in this context, also refer to chapter *Software Autofocus using LSM Tracks [▸ 510]*.

Parameter	Description
Sampling	Here you can select the step size of how the search range is sampled.
- Default	Uses the default step size (**dz = 1/sqrt(2) * 2 * n* lambda/NA**).
- Fine	Uses a small Z-distance (**0.5 * dz**) between the individual focus images that are used to calculate the best focus position. This doubles the number of z-slices for the given range.
- Medium	Uses a medium Z-distance (**2 * dz**) between the individual focus images that are used to calculate the best focus position. Halves the number of z-slices for given range.
- Coarse	Uses a large Z-distance (**4 * dz**) between the individual focus images that are used to calculate the best focus position. Reduces number of z-slices by a factor of four.
Autofocus Search Range	Here you can switch between two distinct approaches for the autofocus search range:
- **Relative Range**	This is the default mode. If selected, the software autofocus is calculated over a relative range. To learn more, read Relative Autofocus Search Range.
- Automatic Range	**Activated:** Calculates the range for the autofocus search automatically depending on the objective set.
- Range	Only active if the **Automatic Range** checkbox is deactivated. Here you can enter a range that you want to be used for the autofocus search.
- Step Size	Shows the distance between the individual focus images set under **Range**.
- **Fixed Range**	If selected, the software autofocus is calculated over a fixed range. To learn more, read Fixed Autofocus Search Range.
- Set Last	Defines the current Z-position as the end (last) point for the software autofocus. Alternatively, you can enter the desired value in the input field to the left of the button.
- Range	Displays the area which is used for the autofocus search. Adopt the area via the Set Last/Set First buttons or the input fields.
- Step Size	Displays the selected **Sampling** distance between the individual focus shots.
- Set First	Defines the current Z-position as the start (first) point for the software autofocus. Alternatively, you can enter the desired value in the input field to the left of the button.
Autofocus ROI	Here you can define a Spot Meter or Focus ROI such that the pixels evaluated by the SWAF are limited to a user defined region of the image.

Parameter	Description
	This is particularly usefully if you use a fiducial marker such as a speck of dirt or other constant artefact that serves as a reference for the sample focus at a fixed position over time. The autofocus ROI is displayed in the live image of the sample enabling it to be positioned and resized as necessary. As an additional aid to help focusing you can also use the focus bar function of the live image that monitors the image contrast in the live image or Spot Meter ROI.
- Spot Meter / Focus ROI	**Activated:** Only uses the values from the Spot Meter / Focus-ROI to calculate the focus position. The Focus-ROI is displayed in the live image as an red dashed rectangle. You can adopt it by clicking on the frame and changing its size and position. Note that this option is not available for LSM acquisition.

12.18.5.2 Software Autofocus using LSM Tracks

Confocal Tracks are also suitable as reference **Channels** for the **Software Autofocus**. As LSM acquisition is by design slower compared to **Camera** acquisition, some optimizations are done in the background in order to speed up the focusing action.

The typical mesasure for the correct focus position in confocal images is the intensity. Hence the aim of the SWAF is here not to generate images of a certain quality, but only to evaluate relative image intensities along the z-stack. This allows us to use very coarse scanning parameters.

Generally, the SWAF for LSM uses a fixed **Frame Size** of 64*64 pixels in combination with the fastest possible scan speed at the currently configured zoom. To further speed up the acquisition, bidirectional scanning is used. Whatever **Laser power** you specify in the **Channels** tool window for this Track is used during the SWAF action. Of note, while you assing a reference Channel, the corresponding Track with all its channels will be active during the focusing.

Some behavior depends on the selected **Search Mode Full** or **Smart**.

In the **Full** search, the system will use the detector gain as configured in the **Channels** tool window.

In contrast, the **Smart** search aims to start close to the likely intensity maximum of the z-Stack. This focus position is approximated by a fast line z-stack in the center of the image frame. As the line scan generates less pixels and a higher noise level, a useful dynamic range needs to be ensured. To this end, the fast line z-stack is repeated several times with increasing PMT gain. After the line scan, regardsless if an intensity peak was found or not, a frame wise autofocus will follow.

In case no peak could be indentified, e.g. because of a sparsely distributed sample, the **Smart** search will start at the original z-position and not optimization of the starting position will take place. Essentially, the **Smart** search will outperform the **Full** search on high **Search Ranges** and a highly varying effective focus position.

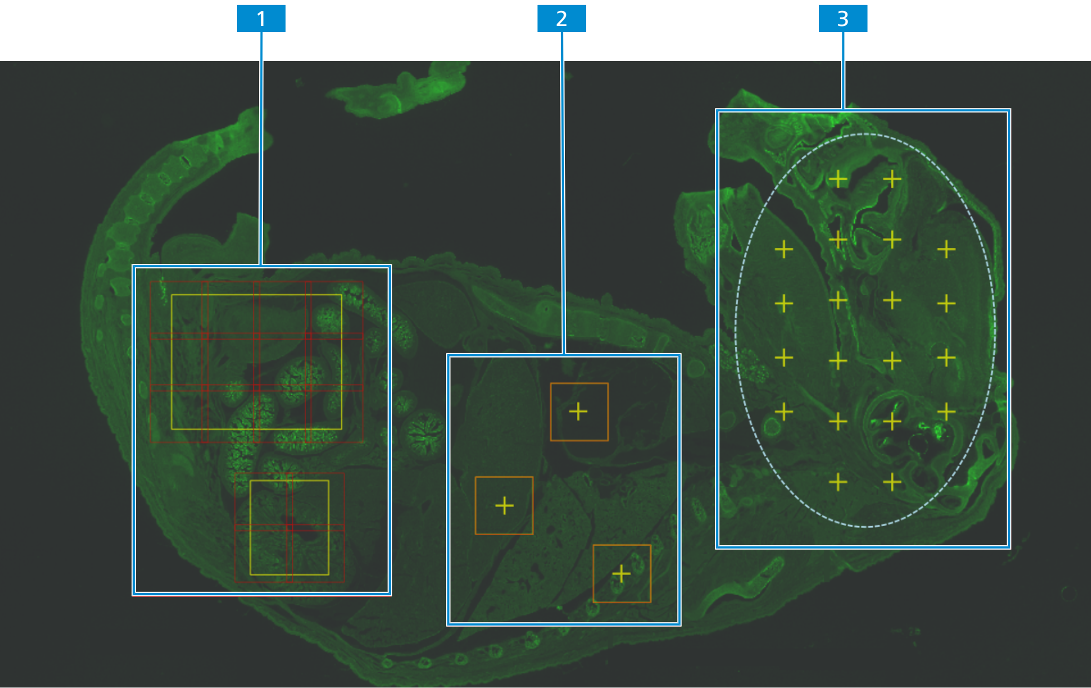
If the focus fluctuations are preditably small, a narrow **Search Range** in combination with a **Full** search might be faster. As a final remark, Camera-based Autofocus can be time-saving, especially when the search range needs to be large and a Full Search is required. While Camera and LSM cannot be combined into one image document, the deactivated camera Track may be still be used as a reference Track.

12.19 Tiles & Positions

This module enables you to acquire images that are made up of a number of individual images (tiles). To do this, it is possible to define tile regions and positions.

If you have licensed the **Tiles & Positions** module, the basic functions of the **Tiles** tool will be supplemented by the **Advanced Setup** functionality. This will allow you to set up **Tiles** experiments more easily and also to use sample carriers and focus surfaces.

Note that when you activate the **Tiles** tool in any experiment for the first time, the software will automatically select the suitable focus strategy **Use Z Values/ Focus Surface defined in Tiles Setup**. This focus strategy also is only available if you have licensed the **Tiles & Positions** module and it can be optimized with the *Focus Strategy Wizard [▸ 716]* in the *Focus Strategy Tool [▸ 711]*. In the case that you do not have the **Tiles & Positions** module, ZEN will still select the appropriate focus strategy for you.



1	**Tile Regions**
2	**Positions (Single Positions)**
3	**Position-Array**

Info

If you want to acquire tile regions or positions with different z-positions, you need to use a suitable focus strategy. To find out more read the chapters *Using focus strategies [▸ 86]*.

See also

- Introduction [▸ 86]
- Set up a new experiment [▸ 44]
- Acquiring Multi-Channel Images with Cameras [▸ 43]

12.19.1 General Preparations

Prerequisite

- ✓ The Tiles module is activated under **Tools > Modules Manager... > Tiles & Positions**.
- ✓ To set up **Tiles** experiments, you require a motorized stage. This must be configured and calibrated correctly in accordance with the camera orientation. For more information read *Calibrating the stage and selecting the channel [▸ 512]*.
- ✓ You are on the **Acquisition** tab.
- ✓ You have *created a new experiment [▸ 44]*, *defined at least one channel [▸ 43]* and correctly set the focus and exposure time.

1. Activate the **Tiles** checkbox in the **Acquisition Dimensions** section to display the **Tiles** tool.
   → In the **Left Tool Area** the **Tiles** tool appears in the **Multidimensional Acquisition** tool group.

You have successfully completed the general preparations. You can now continue with the next steps of this guide.

See also

Setting up a simple tiles experiment without the Tiles & Positions module [▸ 513]

12.19.2 Calibrating the stage and selecting the channel

On start up of a system with motorized stage and/or focus a request will appear asking if the components should be driven to the end switches and calibrated. This ensures that you begin working with absolute coordinates in this session with the microscope. If the microscope power is cycled then this process should be repeated. This function is of particular use if you continually work with a sample carrier e.g. 96 well plate, of the same format mounted in the same manner repeatedly with a given experiment template. If you perform a carrier calibration once with a calibrated stage, then the carrier calibration is essentially always valid. This is done in the **Sample Carrier** section of the **Tiles** tool. Note these features are only available if you have a **Tiles and Positions** license.

Info

The request to calibrate stage and focus on Startup can be activated/deactivated under **Tools > Options > Startup/Shutdown > Stage/Focus Calibration**.

1. Put your Sample Carrier on the stage.
2. Go to the **Acquisition** tab.
3. Choose a low magnification objective (e.g. 10x) from the **Microscope** tool in the **Right Tool Area**.
4. Click on the **Live** button and find your focus area either using transmitted or fluorescence light.
5. In the **Stage** tool of the **Right Tool Area**, activate the **Show all** mode and then click on **Calibrate**.

6. Check if the alignment and orientation of your camera and joystick is correct by dragging the software joystick up, down, left and right and observe whether the movement of your image corresponds to movement of the circle.
   The alignment of the camera is correct if the movement of a given stage axis is congruent to the corresponding axis of the image. An offset in the alignment will be seen as a saw tooth pattern along the edge of a tiled image (e.g. 4x4 tile region).
   In addition, check whether the image movement also corresponds accordingly when you move the hardware joystick of the stage.
7. If the orientation of the camera to the software joystick (stage tool, right tool area) is incorrect, go to the **Camera** tool, activate the **Show all** mode, and click on **Model Specific**. The orientation of the camera (image) can be adjusted by flipping, rotating, or mirroring.
8. Alternatively, you can and may also need to invert the x- and y-axis of your stage in the **MTB** in order to align the hardware joystick and the software-controlled stage movement.
9. Ensure that all the prerequisites (e.g. channel and camera settings) for a **Tiles & Positions experiment** on your sample are fulfilled. If necessary, use the**Smart Setup** for the setup.
10. After you have defined at least one channel (e.g. EGFP), activate the **Tiles** checkbox.
11. If you wish to work with a sample carrier, complete the following steps. Open the **Tiles** tool in the **Multidimensional Acquisition** section and activate the **Show all** mode.
12. In the **Tiles** tool, open the **Sample Carrier** section.
13. Click on **Select...**.
14. Select a predefined **Sample Carrier template** and click on **OK**.

12.19.3 Setting up a simple tiles experiment without the Tiles & Positions module

Prerequisite ✓ You are on the **Acquisition** tab in the **Tiles** tool.

1. Start the **Live** mode to use the stage to locate a point that you want to be at the center of your tile region.
2. Bring the specimen into focus using the focus drive.
3. Open the **Tile Regions** section.
4. The **Tiles** mode is activated by default. In this mode enter the number of tiles you want in the **X** and **Y** input fields, e.g. **X** = 3, **Y** = 3 equals a tiles region containing 9 tiles. Alternatively, you can enter the size of the tile region that you want to add. To do this, activate the **Size** mode.
5. Click on **Add** .
   → The tile region is added to your experiment. The current stage position and focus determines the center and the z-position of the tile region.
6. To add further tile regions, move the stage to another position on the sample and repeat the previous steps.
   → The added tile regions (**TR1**, **TR2**, etc.) are displayed in the tile regions list.
   → If you scroll to the right in the table, you can read the Size of the tile regions.
7. Save the experiment. To do this, in the **Experiment Manager** click on **Options** and select **Save As**. Enter a name for the experiment in the input field (e.g. Simple Tile Experiment).
8. Click on the **Start Experiment** button.
   → The **Tile Region** experiment is acquired.
   → The individual tile regions are displayed in the acquired file as scenes and can be selected using the **Scene** slider on the **Dimensions** tab. If you deactivate the **Scene** checkbox, all tile regions are displayed as an overview.

You have successfully set up and acquired a simple **Tile Region** experiment.

NOTICE

Shortcut

You can also add a predefined tile region at the current stage position by pressing the *F9* button on your keyboard. The size of this region is the last defined number of tiles in x and y, or a square of 3x3 tiles if you have never defined a region before.

12.19.4 Setting up a simple positions experiment without the Tiles & Positions module

Prerequisite ✓ You are on the **Acquisition** tab in the **Tiles** tool.

1. Open the **Positions** section.
2. Start the **Live** mode to use the stage to locate a position that you want to acquire.
   → The X and Y coordinates of the current position are displayed in the **Current X/Y** display fields.
3. Bring the specimen into focus using the focus drive.
4. Click on the **Add** .
   → The current position is added to your experiment.
5. To add further positions, move the stage to another position on the sample and repeat the previous steps.
   → The added positions are shown in the list in the **Single Positions** section with their X, Y and Z-coordinates.
6. Save the experiment. To do this, in the **Experiment Manager** click on **Options** and select **Save As**. Enter a name for the experiment in the input field (e.g. Simple Tile Experiment).
7. Click on the **Start Experiment** button.
   → The Positions experiment is acquired.
   → The individual positions are displayed in the acquired file as scenes and can be selected using the **Scene** slider on the **Dimensions** tab. If you deactivate the **Scene** checkbox, all positions are displayed simultaneously as an overview.

You have successfully set up and acquired a Positions experiment.

NOTICE

Shortcut

You can also add a single position at the current stage position by pressing the *F10* button on your keyboard.

12.19.5 Tiles & Positions with Advanced Setup

Advanced Setup makes it easier for you to create tile regions and positions by displaying the distribution and dimensions of tile regions and positions in the travel range of the stage. You can generate a **Preview Scan** and drawn in tile regions or positions precisely on the basis of this template. For the preview scan you have the option of using an objective with a lower magnification and/or a different channel (e.g. transmitted light).

Info

To ensure that the individual z values of the tile regions and/or positions are taken into account, ZEN automatically selects the most appropriate *focus strategy [▸ 86]* when the checkbox **Tiles** is activated. For the experiment described here no further modification needs to be made. If you want to acquire all tile regions at the same z-position then you must select **None** from the dropdown list in the **Focus Strategy** tool. The individual z-positions are then ignored and the current z-position at the time the experiment is started is used for all tile regions. In the case that you do not have the **Tiles & Positions** module, ZEN will still select the appropriate focus strategy for you.

Prerequisite

- ✓ To set up tiles experiments in **Advanced Setup**, you need the **Tiles & Positions** module.
- ✓ You have read the chapter the general introduction of *Tiles & Positions [▸ 511]*.
- ✓ You are on the **Acquisition** tab in the **Tiles** tool.

1. Click on the **Show Viewer** button.
   - → The **Tiles Advanced Setup** view opens. For more information, see *Tiles Advanced Setup [▸ 544]*.
   - → The live mode is activated automatically. Deactivate the live mode if you do not need it to prevent bleaching of the sample. To do this, click on the active **Stop** button in the **Left Tool Area**. This default behavior can be changed in **Tools > Options > Acquisition > Tiles & Positions**.

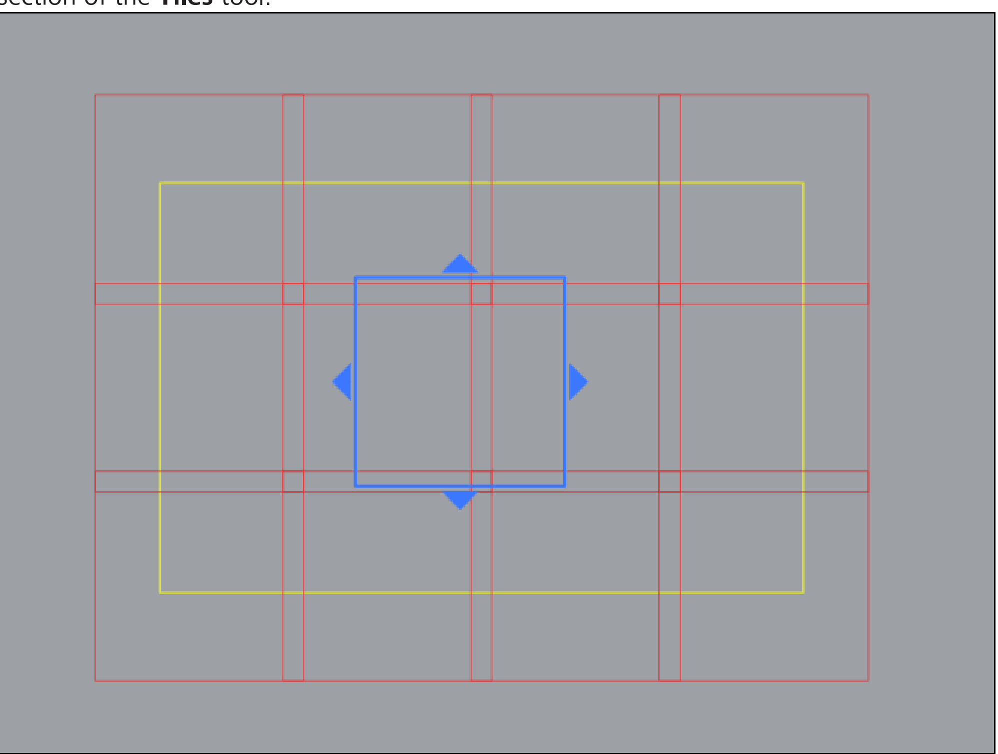
12.19.5.1 Generating a Preview Scan

Prerequisite

- ✓ You have selected an objective with a relatively low magnification in your experiment settings.
- ✓ You are in **Advanced Setup**.

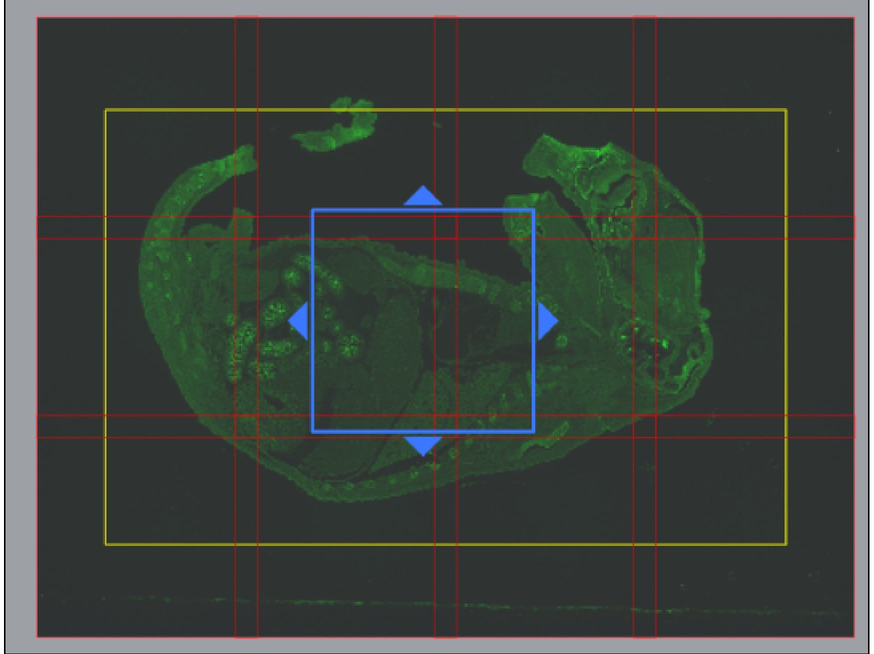
1. In the left toolbar, click on **Preview Scan**.
   - → The *Preview Scan Toolbar [▸ 548]* is displayed as the top toolbar.
2. In the top toolbar, deactivate **Use Existing Experiment Settings** and select/ unselect the channels that you want to use for the preview scan.
3. If necessary, use the live mode to adjust the focus area and exposure following a change of objective or channel.
4. To obtain a better overview, slightly zoom out of the **Advanced Setup** view.
5. Start the **Live** mode to use the stage to locate approximately the center of the region for which you want to generate a preview scan.
6. In the *left toolbar [▸ 547]* in the **Tiles** section, click on the **Setup by contour** button.
   - → The *Contour Toolbar [▸ 548]* is displayed as the top toolbar.
7. In the top toolbar, select the **Rectangular Contour** tool.
8. In the Stage View, use the tool to draw a rectangle that approximately encloses the region for which you want to generate a preview scan.

9. Alternatively, in the Stage View, use the live image to navigate to the edge of the area that you want to image. We recommend, for example, the bottom left corner. Add the first marker. Now navigate to the upper edge of the object and add a second marker. Finally, if necessary, navigate to the right edge and add a third marker and click on **Done**. You can optimize the tile region by selecting a circular contour shape for your sample instead.
   → A tile region is created for the marked region and displayed in the list in the **Tile Regions** section of the **Tiles** tool.



10. With the help of the **Live** mode, check whether the desired image region is covered by the tile region. To do this, use the stage to locate the corners and edges of the tile region and increase or reduce the yellow selection frame as necessary.
11. In the left toolbar, click on **Preview Scan** and in the top toolbar click on **Start**.

   → A series of snap images is acquired to generate a preview of the marked region.



You have successfully generated a preview scan.

Before you continue with the actual experiment, carry out the following steps:

1. In the **Tiles Regions** section, deactivate the preview tile region (TR 1) by deactivating the checkbox of the corresponding list entry. This prevents the acquisition of the preview tile region during the actual experiment.
2. In the **Microscope Control** tool in the **Right Tool Area**, select the objective you want to use for final acquisition.
3. Use the **Live** mode to adjust the focus area and exposure accordingly.

You can now continue setting up the tile experiment.

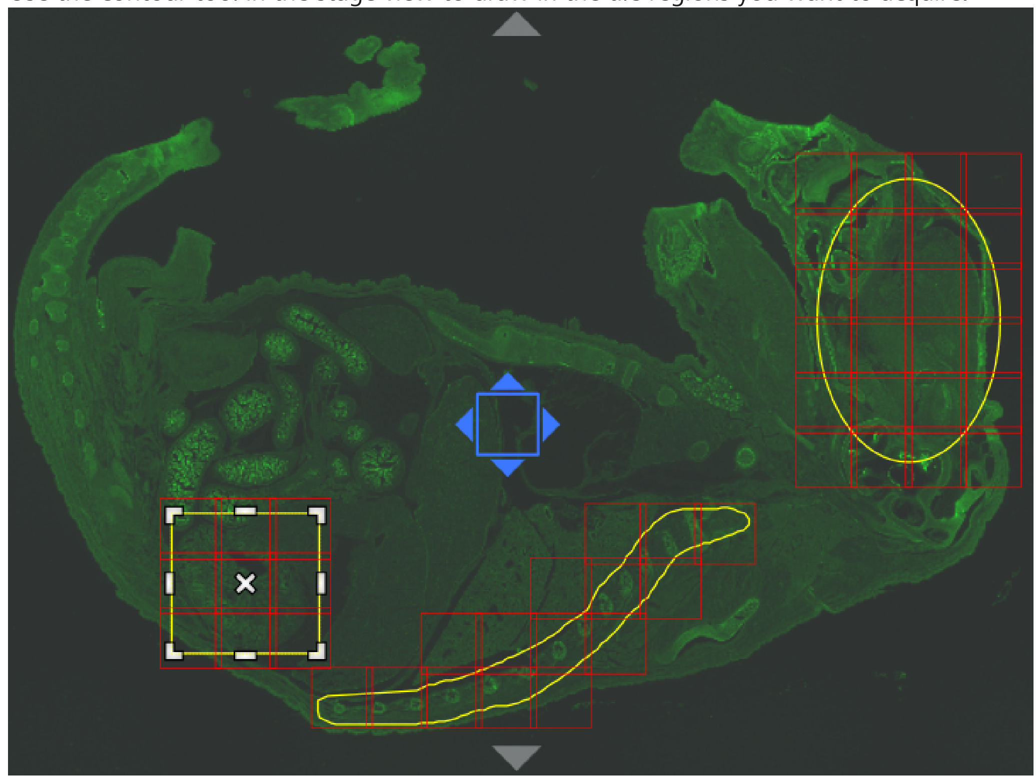
See also

- Channels Tool [702]
- Tiles Advanced Setup [544]
- Tiles & Positions with Advanced Setup [515]

12.19.5.2 Creating Tile Regions by Contour

Prerequisite ✓ You have generated a *preview scan [515]* that will help you to position the tile regions more easily.

1. In the *left toolbar [547]* in the **Tiles** section, click on the **Setup by contour** button.
   → The *Contour Toolbar [548]* is displayed as the top toolbar.
2. In the top toolbar, select the desired contour tool.
3. Use the contour tool in the stage view to draw in the tile regions you want to acquire.



   → Tile regions are created for each marked region. They are added to the list in the **Tile Regions** section of the **Tiles** tool.

You have successfully created tile regions in **Advanced Setup**.

12.19.5.3 Creating Tile Regions by Predefined

1. In the *left toolbar [▸ 547]* in the **Tiles** section, click on the **Setup by predefined** button.
   → The *Predefined Toolbar [▸ 549]* is displayed as the top toolbar.
2. In the top toolbar, choose how many tiles in x and y dimension you want to add.
3. Click on **Add Tile Region** to add the respective tile region.

You have now created tile regions in the **Advanced Setup**.

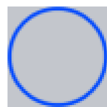
NOTICE

Shortcut

You can also add a predefined tile region at the current stage position by pressing the *F9* button on your keyboard. The size of this region is the last defined number of tiles in x and y, or a square of 3x3 tiles if you have never defined a region before.

12.19.5.4 Creating Tile Regions by Carrier

Prerequisite ✓ You have selected and calibrated a sample carrier with one or more wells/ containers.

1. In the *left toolbar [▸ 547]* in the **Tiles** section, click on the **Setup by carrier** button.
   → The *Tiles Carrier Toolbar [▸ 550]* is displayed as the top toolbar.
2. In the **Carrier** tab, select the individual wells for which you want to create tile regions by pressing the *Ctrl* key and clicking on the desired wells.
   → The selected wells are now bordered by a blue circle.
   
3. In the top toolbar, select **Fill Factor** and enter the desired value in the **Fill Factor** input field.
4. Click on .

According to the selected **Fill Factor**, the wells will be filled with a calculated number of tiles that are located around the center. To create a given size of tile region, use the **Columns/Rows** function in a similar manner.

12.19.5.5 Creating Positions by Location

1. In the *left toolbar [▸ 547]* in the **Positions** section, click on the **Setup by location** button.
   → The *Positions Location Toolbar [▸ 551]* is displayed as the top toolbar.
2. In the top toolbar, click on the **Add** button .
3. In the stage view, click on the location where you want to add a position.
   → The added positions are displayed in the stage view and the **Single Positions** list in the **Positions** section of the **Tiles** tool.

You have successfully created positions in **Tiles- Advanced Setup**.
Note: ZEN will query if you want to add a position that has more than 66 % overlap of the field of view in either x or y with an existing position.

NOTICE

Shortcut

You can also add a single position at the current stage position by pressing the *F10* button on your keyboard.

12.19.5.6 Creating Positions by Array

1. In the **Tiles** tool, open the **Positions** section and click on **Position Arrays**.
2. In the *left toolbar [▶ 547]* in the **Positions** section, click on the **Setup by array** button.
   → The *Position Array Toolbar [▶ 551]* is displayed as the top toolbar.
3. In the top toolbar, choose either the rectangular or circular **Contour**, adjust the **Number** of required positions and the **Bias** where the positions should be located.
4. Mark the interesting area in the **Center Screen Area** with a pressed left mouse button.

The positions will be generated automatically.

12.19.5.7 Creating Positions by Carrier

Prerequisite
- ✓ You have selected and calibrated a *sample carrier template [▶ 529]*.
- ✓ You are on the **Acquisition** tab.

1. To obtain a complete overview of the sample carrier, zoom out of the view using the mouse wheel. If necessary, use the panning tool (press *Alt* and the left mouse button) to move the stage view as needed.
2. In the *left toolbar [▶ 547]* in the **Positions** section, click on the **Setup by sample carrier** button.
   → The *Positions Carrier Toolbar [▶ 552]* is displayed as the top toolbar.
3. Select the containers in which you want to distribute positions by pressing the *Ctrl* key and clicking on the relevant containers.
4. In the top toolbar adjust the **Number** of required positions and the **Bias** where the positions should be located. Alternatively, if you want to create the positions as a **Grid**, adjust the **Columns**, **Rows**, and **Overlap**.
5. Click on the **Create** button [+].
   → The selected containers are each filled with a **Position Array** (group of positions).

→ In the **Positions** section of the **Tiles** tool, the **Position Arrays** are displayed in the **Position Arrays** list.

You have successfully used a sample carrier to create positions.

12.19.6 Copying a Tile Region or Position

To copy and paste a specific Tile Region or Position, follow these instructions.

1. Right-click the respective tile region or position and select **Copy**. Alternatively, press *Ctrl+C*. If you want to copy multiple tile regions and positions, select multiple regions or positions by dragging a selection box across the objects or click to select multiple objects while pressing the *Ctrl* button.
2. Right-click in the setup and select **Paste**. Alternatively, press *Ctrl+V*.

The copied tile regions/ positions are now pasted next to the originally copied regions/ positions.

Copying Tile Region or Position settings between wells

When you want to copy and paste a tile region or position setting (e.g. a certain arrangement of tiles, positions or local support points) from one well to other wells or even to all containers of a carrier, apply the following workflow.

1. Select the well from where the tile region/ position setting should be copied.
   → The selected well is now highlighted by a blue border.
2. Right click within the selected well in the **Center Screen Area** (outside the tile region) to open the context menu.
3. Select **Copy Container for replication**.
4. If you want to choose specific wells use the left mouse button to select the wells into which you want to paste the copied tile region/ position setting.
5. Right click in the **Center Screen Area** and select the context menu entry **Paste Replication to** and either choose **Selected Container** or **All Container**.

The copied tile region/ position setting is pasted into the selected wells or all the wells of the carrier with the same relative coordinates to the center of each well.

12.19.7 Adjusting Z-Values

If you add positions or tile regions, the current Z-value is automatically adopted for the tile region or position.

- Learn about how to adjust and verify the Z-values of positions in the chapter *Adjusting Z-Values of Positions [▸ 521]*.
- Learn about how to adjust and verify the Z-values of tile regions in the chapter *Adjusting Z-Values of Tile Regions [▸ 521]*. Note that the Z-values defined here are valid for all tiles in the respective tile region.

Info

To ensure that the individual z values of the tile regions and/or positions are taken into account, ZEN automatically selects the most appropriate *focus strategy [▸ 86]* when the checkbox **Tiles** is activated. For the experiment described here no further modification needs to be made. If you want to acquire all tile regions at the same z-position then you must select **None** from the dropdown list in the **Focus Strategy** tool. The individual z-positions are then ignored and the current z-position at the time the experiment is started is used for all tile regions. In the case that you do not have the **Tiles & Positions** module, ZEN will still select the appropriate focus strategy for you.

See also

Creating a Local Focus Surface [▸ 522]

12.19.7.1 Adjusting Z-Values of Tile Regions

Prerequisite ✓ You have set up a **Tiles** experiment with at least one tile region.

1. To check the z-value of tile regions, open the **Tile Regions** section in the **Tiles** tool.
   → The z-values of the tile regions are displayed in the **Z** column of the list.
2. Double-click on the list entry of the tile region that you want to check.
   → The stage automatically locates the center of the tile region and the associated z-position.
3. Use the **Live** mode to check the z-value of the tile region.
4. To adjust the z-value, set the new z-position with the **Focus** tool.
5. In the **Tile Regions** list, click on **Options** and select **Set Current Z For Selected Tile Regions**. Alternatively, in the **Tile Regions** list, right-click the tile region entry and select **Set Current Z For Selected Tile Regions**.
6. To check further tile regions, repeat steps 2 to 4.
7. To check and adjust large number of tile regions, click on the **Verify** button.
   → The **Verify Tile Region** dialog opens. There you have a interface for the verification process of each tile region.
8. Click on **Close** after you have verified all tile regions.

You have successfully checked and adjusted the individual z-values for the tile regions.

See also

Introduction [▸ 86]

12.19.7.2 Adjusting Z-Values of Positions

Prerequisite ✓ You have set up a tile experiment with at least one position.

✓ You are on the **Acquisition** tab in the **Tiles** tool.

1. To check and adjust the z-value of positions, open the **Positions** section.
   → The z-values are displayed in the **Z** column of the **Single Positions** list.
2. Double-click on the list entry of the position that you want to check.
   → The stage automatically locates the position.
3. Use the **Live** mode to check the z-position of the position.
4. To adjust the z-value, set the desired position using the focus drive.

5. In the **Single Positions** list, click on **Options** and select **Set Current Z For Selected Positions**. Alternatively, in the **Single Positions** list, right-click the position entry and select **Set Current Z For Selected Positions**.
6. To check and adjust a large number of positions, use the **Verify Positions** dialog.
7. To do this, click on the **Verify Positions** button in the **Positions** section.
   → The **Verify Positions** dialog opens.
8. Select the **Helper Method** you want to use. This will support you in determining the z-values. The options are **Autofocus (AF)** and **Definite Focus (DF)**. If you have neither then you can only adjust z-values manually.
9. Click on the **Move to Current Point** button.
   → The stage moves automatically to the position in the list that is highlighted in blue. Alternatively, you can double-click on the position in the list that you want to check.
10. In the **Live** mode use the **Focus** (or **SW Autofocus**) tool to adjust the desired z-value.
11. Click on the **Set Z and Move to Next** button.
    → The position is marked with a check mark.
    → The stage moves automatically to the next position in the list.
12. Repeat the last 3 steps until you have checked all the points in the list.
    → The message **All points have been verified** appears.
13. Close the **Verify Positions** dialog.

You have successfully verified and adjusted the individual z-values for positions.

See also

Introduction [86]

12.19.8 Local and Global Focus Surfaces

12.19.8.1 Introduction

Local Focus Surface

To acquire large tile regions on tilted or uneven specimens, you need to assign individual z-values to each tile of a tile region efficiently. ZEN will help you do this by creation of a **Local Focus Surface**. Note that a local focus surface is always associated with precisely one tile region. Thus, you need to create a focus area separately for each tile region.

Global Focus Surface

To create a focus area covering the entire sample, you need to create a **Global Focus Surface**. Global focus surfaces are based on a sample carrier template (e.g. for slides or multiwell plates) and result in a focus surface that is valid for the entire sample carrier and therefore for all the tile regions and positions it contains. This allows you to compensate for any tilting and curvature of the sample carrier.

12.19.8.2 Creating a Local Focus Surface

To create local focus surfaces, you must distribute support points across your tile regions and assign their focus position. Tile-region-specific focus values are then interpolated to generate a focus surface that approximates the topology of the area you want to image.

12.19.8.2.1 Distributing Support Points

Prerequisite
- ✓ To create a local focus surface you need the **Tiles & Positions** module.
- ✓ You have set up a **Tiles** experiment with at least one tile region.
- ✓ You are on the **Acquisition** tab in the **Tiles** tool.

1. Click on the **Show Viewer** button.
   → The advanced tile setup opens.
2. Select a tile region for which you want to create support points. To do this, click on the corresponding tile region in the list in the **Tile Regions** section of the **Tiles** tool. Alternatively, you can select tile regions by clicking directly on the desired tile region in the **Advanced Setup** view. Both methods allow you to select several tile regions when pressing the *Ctrl* key.
3. Open the **Focus Surface and Support Points** section in the **Tiles** tool.
4. To add a single support point at the current stage position, click on **Current Position**. Alternatively, you can add a single support point to the center of the currently selected tile region by clicking on **Center of Tile Region**.
5. Under **Add Multiple Support Points**, you have the settings to add multiple support points. Indicate the number of **Columns** and **Rows** for the distribution of the reference points. Alternatively, recommended for larger tile regions (>200 tiles), you can use the distribution method **Onion Skin**. Depending on the total size and shape you might need to adjust the density parameter and/ or the maximum number of support points to optimize the result. Typically, this method works best with large irregular or rounded tile regions.
6. Click on **Distribute**.
   → The support points are distributed within the selected tile region(s) and shown as yellow points in the stage view.
   → The support points of the selected tile region are displayed with their coordinates in the **Local (per Tile Region)** list in the **Focus Surface and Support Points** section of the **Tiles** tool.
7. If necessary, you can adjust the distribution of the support points manually in the **Tiles - Advanced Setup**. You can change the position of the support points using drag & drop.
8. Repeat the steps until you have distributed reference points across all desired tile regions.

You have successfully distributed support points across the tile regions.

12.19.8.2.2 Verifying Z-Values of Support Points

1. Click on the **Verify** button in the **Focus Surface and Support Points** section of the **Tiles** tool.
   → The **Verify Tile Regions / Positions** dialog opens.
2. Select the **Helper Method** you want to use. This will support you in determining the z-values. The options are **Autofocus (AF)** and **Definite Focus (DF)**. If you have neither then you can only adjust z-values manually.
3. Click on the **Move To Current Point** button.
   → The stage automatically moves to the support point that is highlighted in the reference point list. Alternatively, you can also double-click on the support point you want to check in the list .
4. In the **Live** mode use the **Focus** tool to adjust the z-value.
5. Click on the **Set Z and Move to Next** button.
   → The checked reference point is marked with a green check mark.
   → The stage moves automatically to the next support point in the list.
6. Repeat the last 3 steps until you have checked all the support points.

→ The message **All points have been verified** appears.
7. Close the **Verify Tile Regions/Positions** dialog.

You have adjusted and verified the z-values of all support points.

Info

Positions always have a focus, which is determined by the z-value of the position. If you use positions in addition to tile regions, you can verify the z-values of the positions with the help of a similar dialog. Open this dialog by clicking on the **Verify** button in the **Positions** section of the **Tiles** tool.

12.19.8.2.3 Selecting Interpolation Degree

1. If necessary, select the interpolation level in the **Interpolation Degree** dropdown list in the **Focus Surface and Support Points** section of the **Tiles** tool.

You have successfully created a local focus surface. You can now start the experiment. To ensure that the tiles are acquired along the focus surface the software automatically selects the most appropriate focus strategy. For more information on focus strategies read the chapter *Working with Focus Strategies [▶ 86]*.

Info

The minimum number of support points necessary per tile region is indicated in the **Interpolation Degree** dropdown list for each entry. The calculation is more solid if the number of support points exceeds this minimum number. We therefore recommend that you only increase the interpolation degree as far as the surface of the sample demands, even if you have set more support points. If the number of support points does not correspond to the minimum number for the selected interpolation degree, the interpolation degree will be reduced automatically. By default, ZEN uses a second order interpolation degree that creates a parabolic focus surface and requires at least 9 support points. Typically, this will be suitable for many samples and imaging scenarios.

12.19.8.3 Creating a Global Focus Surface

To create a global focus surface, you must distribute support points across your sample carrier and define their focus position. A focus area across the sample carrier is then interpolated from the values of these reference points.

12.19.8.3.1 Distributing Support Points

Prerequisite

- ✓ You have configured the general settings for setting up a tile experiment (experiment created, at least one channel defined, **Tiles** dimension activated).
- ✓ To create a global focus surface, you need the **Tiles & Positions** module.
- ✓ You are on the **Acquisition** tab in the **Tiles** tool.

1. Open the **Sample Carrier** section.
2. Click on the **Select...** button.
   → The **Select Template** dialog opens.
3. Select the sample carrier template that you want to use.
4. Click on **Options** and select **Copy And Edit...**.

→ A copy of the existing template is generated and opened in the sample carrier editor.

5. To distribute support points across the sample carrier template, open the **Global Support Points** section.
6. Select the containers in which you want to create global support points. To do this, press the *Ctrl* key and click on the containers.
7. Click on the **Distribute One Support Point For Each Selected Container** button.
   → One support point is assigned to each selected container.
   → The support points are distributed automatically across the sample carrier.
   → You can add further support points manually using the **Add** button .
8. To close the **Editor** window, click on **OK**.
9. To select the edited sample carrier template, click on **OK**.
   → If you wish to re-edit the global support points at any time, click on the **Edit** icon in the sample carrier section to open the sample carrier editor again.
10. To calibrate the sample carrier, click on the **Calibrate** button and follow the wizard.

You have successfully distributed support points across a sample carrier template and have selected and calibrated it.

12.19.8.3.2 Verifying Z-Values of Support Points

1. In the **Tiles** tool open the **Focus Surface and Support Points** section.
2. Go to the **Global (on Carrier)** tab.
   → All the support points of the selected sample carrier template are displayed in the **Support Points** list.
3. Click on the **Verify** button.
   → The **Verify Global Support Points** dialog opens.
4. Select the **Helper Method** you want to use. This will support you in determining the z-values. The options are **Autofocus (AF)** and **Definite Focus (DF)**. If you have neither then you can only adjust z-values manually.
5. Click on **Move To Current Point**.
   → The stage automatically moves to the support point that is highlighted in the list. Alternatively, you can also double-click on the support point in the list.
6. In the **Live** mode use the **Focus** (or **SW Autofocus**) tool to set the z-value.
7. Click on **Set Z and Move to Next**.
   → The support point is marked with a check mark.
   → The stage automatically moves to the next support point in the list.
8. Repeat the last 3 steps until you have checked all the support points.
   → The message **All points have been verified** appears.
9. Close the **Verify Global Support Points** dialog.

You have adjusted and verified the Z-values of all support points.

12.19.8.3.3 Selecting Interpolation Degree

1. Select the interpolation degree in the **Interpolation Degree** dropdown list in the **Focus Surface and Support Points** section.

You have successfully created a global focus surface.

You can now set up your tile experiment using the sample carrier. Further information on this can be found under: *Using Sample Carriers [▸ 529]*. To ensure the tiles are acquired along the focus surface during the experiment the software automatically selects the most appropriate focus strategy in the **Focus Strategy** tool. For information on focus strategies read the chapter *Working with Focus Strategies [▸ 86]*.

Info

The minimum number of support points necessary is indicated in the **Interpolation Degree** dropdown list for each entry. The calculation is more solid if the number of support points exceeds this minimum number. We therefore recommend that you only increase the interpolation degree as far as the surface of the carrier demands, even if you have created more support points. If the number of support points does not correspond to the minimum number for the selected interpolation degree, the interpolation degree will be reduced automatically. Interpolation degree **1 – Tilted Plane (at least 4 support points)** is typically sufficient to compensate for any tilting of the sample carrier.

12.19.9 Assigning Categories to Tile Regions and Positions

In some cases it can be helpful to not only display the well number together with the acquired images (Path: **Graphics > Frequent Annotations > Carrier Container Name**) but also to create certain additional annotations for different tile regions or positions, e.g. "control condition" or "experimental condition 1".
For that purpose, the software allows you to add and edit names and categories to the different tile regions/ positions that have been generated.

1. In order to assign individual names to different individual positions and/or tile regions in a well plate experiment, in the **Tiles** tool click on the respective name in the **Tile Regions** or **Positions** list. You can now edit the name field, press *Enter* to finish.
2. Repeat this step to rename different tile regions or positions.
3. To assign or edit categories of your tile regions/ positions, first select all desired tile regions/ positions that should be grouped in the same category.
4. Under the **Tile Regions** / **Positions** list, in the **Properties** section **Category**, click on the options button and select **New** from the dropdown list.
   → The **New Category** window opens.
5. Enter a **Name** and add a **Description** for the selected tile regions/ positions.
6. Assign a **Color** for the new category by clicking on the color bar and choosing a preferred color.
7. Click on **OK** to create the new category.
   → The **New Category** window closes and the new category is created.
8. As **Category** choose the desired category for the selected tile regions/ positions from the drop down list.

The chosen category is now assigned to the selected tile regions/ positions.

Info

- To display the name of your Tile Region/ Position later in your acquired image(s), go to **Graphics > Frequent Annotations > More…** and select **Image.Scene.Name** from the metadata list.
- Note that a predefined category can also be applied to a differentiated selection of Tile Regions/ Positions from more than one well.
  Note also, that the assigned color is only used as a feature in the Tiles tab (Left Tool Bar Area).
- To display a Tile Region/ Position Category feature (**Name** and/or **Description**) in your acquired image, you go to **Graphics > Frequent Annotations > More…**. Type "category" in the search bar and select the desired feature to be displayed. (Although the option "Color" is given, no reasonable element will be displayed by the software)
- To adjust parameters of your annotations (e.g. font size), right-click on it and go to **Format > Graphical Elements**.

Displaying categories in the Tiles/ Positions List (Left Tool Area)

Prerequisite ✓ You have selected several different positions or tile regions and assigned different categories.

1. Under **Positions** or **Tile Regions** of the **Tiles** tool, select a position or tile region.
2. Right-click on the selected position/ tile, choose **Sort** and select **By Category**

The positions/ tiles will be sorted alphabetically according to the assigned categories.

12.19.10 Re-positioning Sample Carrier after Incubation

When you want to take images of positions/ tile regions on a sample carrier, that has to be taken off the stage, e.g. for incubation purposes or changes of the immersion medium, proceed as follows to re-position your sample carrier.

Starting the Experiment

Prerequisite
- ✓ You have run the stage calibration and have located your sample, see chapter *Calibrating the stage and selecting the channel [▸ 512]*.
- ✓ You have set up at least one channel and adjusted the light/ camera exposure time.
- ✓ You have activated the **Tiles** checkbox and the **Show All** mode

1. In the Tiles tool, open the **Sample Carrier** section and click on **Select**.
   → The **Select Template** dialog opens.
2. Select the template of your choice.
3. Adjust the surface of the sample carrier. Refer to the chapter *Creating a Global Focus Surface [▸ 524]*.
4. Click on **OK**.
   → The **Select Template** dialog closes.
5. Click on **Calibrate**.
   → The **Sample Carrier Calibration Wizard** opens.
6. Move a sample reference point into the middle of the crosshair. This reference point can be any unique, identifiable point on the slide and does not have to in the middle of the slide.
7. Click on **Next**.
8. Under **X/Y Position** click on **Set Zero**.

9. Click on **Next**.
10. As Calibration Method, select **Search Reference Point**
11. Click on **Next**.
12. Click on **Set Current X/Y**.
13. In the **Tiles** tool, click on **Show Viewer** and add positions/tile regions at your locations of interest.
14. Once you have defined all your positions/ tile regions, click on **Options** on the **Acquisition** tab to in the **Experiment Manager**.
15. Save your experimental settings, including the lists of positions/ tile regions, by selecting either the **Save As** or **Export** entry.
16. Start your experiment and record images from your selected positions/ tile regions.
17. Remove your sample off the stage and e.g. put it back into the incubation chamber.
18. Close the software.

You have done all settings for a successful re-positioning of your sample carrier after the experiment.

Info

- For demo purposes, select a standard slide that can mimic your test sample.
- Regarding calibration of your template, you can customize your own carrier see the chapter *Customizing a Sample Carrier Template [▸ 529]*, but for slides with one coverslip or well, there is only the option for Single Reference Point Calibration. For Multi-well plates, you will have the option for 7-point, 4-point, 3-point or 1-point calibration. This becomes important for adjusting for the rotation of the sample.
- It is assumed that you just use a conventional glass slide with some cells or tissue that is positioned in the center
- You can zoom in and out using the mouse scroller, and move the stage in the Center Screen Area to a point of interest with a double-click on the sample carrier.
- With **Save As** the settings will be saved directly in the Experiment Manager. With **Export** the settings will be saved in a folder of your choice.

Re-Positioning of the Sample Carrier after the experiment

Info

If you cycle the power on the microscope the software will prompt you to calibrate the stage and/ or focus drives. Thus, if the calibration of the multi-well plate was performed under the same conditions then the sample carrier calibration will still be valid. You must however, ensure that other parameters like plate orientation and placement on the microscope have not changed.

1. Restart the software.
2. In the **Acquisition** tab go to the **Experiment Manager** and **Reload** or **Import** your experimental settings including your list of positions/ tile regions.
3. In **Tiles** tool under **Sample Carrier**, click on **Calibrate**.
   → The **Sample Carrier Calibration Wizard** opens.
4. Move your previously chosen sample reference point into the middle of the crosshair.
5. Click on **Next**.
6. Under **X/Y Position** click on **Set Zero**.

7. Click on **Next**.
8. As Calibration Method, select **Search Reference Point**.
9. Click on **Next**.
10. Click on **Set Current X/Y**.
11. Now, you still need to verify the Z-offset of your positions. Therefore, follow the corresponding instructions given in the chapters *Adjusting Z-Values of Tile Regions [▸ 521]* and *Adjusting Z-Values of Positions [▸ 521]*.

All of your selected positions/tile regions are now re-assigned to the correct X/Y/Z-values in relation to your (unique identifiable) reference point.

You can re-start your experiment and record images from your selected positions/tile regions.

See also

Customizing a Sample Carrier Template [▸ 529]

12.19.11 Using Sample Carriers

Use a sample carrier template to display the size and appearance of your sample carrier (e.g. slide or multiwell plate) in **Advanced Setup**. This allows you to distribute tile regions or positions easily across your sample carrier.

12.19.11.1 Selecting a Sample Carrier Template

Prerequisite

- ✓ You have configured the general settings for setting up a tile experiment (experiment created, at least one channel defined, Tiles dimension activated).
- ✓ You are on the **Acquisition** tab in the **Tiles** tool.

1. Open the **Sample Carrier** section and click on the **Select...** button.
   → The **Select Sample Carrier Template** dialog opens.
2. Select an existing sample carrier template or generate a new template by clicking on .
3. To close the dialog, click on **OK**.
4. Calibrate the sample carrier by clicking on the **Calibrate** button.
   → The **Sample Carrier Calibration Wizard** opens.
5. Follow the wizard until you have fully calibrated the sample carrier.
   → The information **The sample carrier is calibrated** appears in the **Sample Carrier** section.

You have successfully selected a **sample carrier**.

12.19.11.2 Customizing a Sample Carrier Template

If you want work with a sample carrier that is not listed in the template database, you will need to apply the following workflow in order to create a new template.

Prerequisite

- ✓ You have done all prerequisites for a Tiles & Positions experiment
- ✓ You have defined at least one channel.
- ✓ You have activated the **Tiles** checkbox.

1. Go to the **Acquisition** tab.
2. Open the **Tiles** tool and activate the **Show All** mode.
3. Open the **Sample Carrier** section and click on the **Select...** button.

→ The **Select Template** dialog opens.

4. Click on the **Options** and select **New Template**.
   → The **New Sample Carrier Template** dialog opens.
5. Choose a **Category** that corresponds to the type of your carrier and assign a **Name** to your template.
6. For example if you select **Slide**, you can now configure the **Width** and **Height** of the slide and adjust the location of a **Reference Point**.
7. If you select a **Multislide**, **Petri Dish**, **Multiwell** or **Multichamber** template, you can configure and adjust additional parameters of your carrier.
8. In case you need to modify one of the above depicted templates even further, first select the **Category** that appears closest to your carrier, go again to the **Category** tab and then choose **Custom**.

You have customized a sample carrier template or set up a custom one.

12.19.12 Importing Images into the Advanced Setup

In the **Tiles - Advanced Setup**, you have several options to open/import images. Follow the respective workflow.

Importing preview scan images

Prerequisite ✓ You are in the **Advanced Setup**.

1. In the ZEN menu bar, click on **File > Tiles > Import Preview Image**. Alternatively, right click in the viewer and select **Import Preview Image** from the context menu.
   → A file browser opens.
2. In the file browser, select your preview scan image (*.czi) and click on **Open**.

You have now imported a preview scan image into the **Tiles Advanced Setup**.

Importing an image already opened in ZEN

Prerequisite ✓ You are in the **Advanced Setup**.

✓ You have an image opened in ZEN.

1. In the **Right Tool Area**, in the **Images and Documents** tool, select the image you want to import.
2. Drag and drop the image into the viewer of the **Advanced Setup**.

You have now imported an already opened image into the **Tiles Advanced Setup**.

Importing from Explorer

Prerequisite ✓ You are in the **Advanced Setup**.

✓ You have opened the path to your image (*.czi) in the Windows **File Explorer**.

1. In the Windows **File Explorer**, select the image you want to import.
2. Drag and drop the image into the viewer of the **Advanced Setup**.

You have now imported an image from the Windows **File Explorer** directly into the **Tiles Advanced Setup**.

12.19.13 Exporting Tile Images

Prerequisite ✓ You have acquired or opened a tile image.

1. On the **Processing** tab, open the parameters for **Image Export** (*Ctrl+6* or via **File > Export/Import > Export**).
   → You will see the settings of the parameters for **Image Export**. Make sure that the **Show All** mode has been activated.
2. Select the file type that you want to use. We recommend the **PNG** format, as this is a format that offers lossless compression with an acceptable file size.
3. Activate the **Apply Display Curve and Channel Color** checkbox. This means that the images will be exported with the settings you have made, e.g. tonal value corrections or contrast. If you activate the **Original Data** checkbox, the images are exported unchanged. In this case, the settings from the display curve, e.g. tonal value corrections and contrast, are not adopted.
4. Select the **Define Subset** radio button.
   → The settings for the available dimensions open.
5. Open the settings for the **Tiles** dimension.
6. Select the **Existing Tiles** radio button.
7. Click on the **Apply** button at the top of the **Processing** tab.
   → You have exported the individual tiles from a tile image. The files can be found in the export folder indicated.

12.19.14 Functions & Reference

12.19.14.1 Tiles Tool

Info

The basic **Tiles** tool is only visible if you have a motorized stage configured with your microscope. The **Tiles Advanced Setup** and many other functions are only available if you own the **Tiles & Positions** module and when it is activated in the **Modules Manager**. Additionally, you must activate the corresponding checkbox on the **Acquisition** tab in the **Experiment Manager**. This tool is part of the basic license for LSM.

In the **Tiles** tool you configure the acquisition of images that consist of several image fields. Therefore you define Tile Regions or Positions. In addition you can set up focus surfaces and sample carrier templates here.

The **Tiles** tool is located in the **Left Tool Area** under **Multidimensional Acquisition**.

Parameter	Description
Show Viewer	Only available if you have the **Tiles & Positions** license. Opens the *Tiles Advanced Setup [▸ 544]* view in the **Center Screen Area**.

Info

The **Sample Carrier**, **Focus Surface** and **Options** sections are only visible if the **Show All** mode is activated.
If you have no license for the **Tiles** module you will only find **Tile Regions**, **Positions** and **Options** sections here.

The different sections of the tool are described in the next chapters.

12.19.14.1.1 Tile Regions Section

Here you can define the desired tile regions and add it to the image.
Note: This section with controls is only visible if you have no license for **Tiles & Positions**. With a license, these controls are selected from the *Left Toolbar [▸ 547]* in the **Tiles Advanced Setup**.

Parameter	Description
Contour	This parameter is only visible if the **Show All** is activated. Here you select the shape or contour of the tile region that you are adding. Simply click on the corresponding button to select the desired contour. The selected contour is highlighted in blue color.
- Rectangle	If selected, you can create rectangular tile regions.
- Circle	When selected, you can create circular tile regions.
Mode	
- Tiles	If selected, you have to enter the number of tiles as a reference for the size of the tile region. Enter the number of tiles in the **X/Y** input fields. If you are adding a circular tile region, enter the number of tiles for the diameter in the **Diameter** input field.
- Size	If selected, you have to enter the size as a reference for the size of the tile region. Enter the size of the tile region in the **X/Y** input fields. If you are adding a circular tile region, enter the diameter of the tile region in the **Diameter** input field.

Parameter	Description
- Stake	If selected, you can define a tile region by the placement of at least two markers (user defined X/Y stage coordinates). If you want to modify the tile region (expand/reduce) you have to adjust the tile region to the desired size. To complete the tile region press **Done**. Circular or rectangular tile region can be created in this manner by selection of the appropriate contour.
- Add	Adds the tile region to the image. The added tile region will also appear in the **Tile Regions List** and is activated for acquisition. Added tile regions are displayed in the form of red grids in the stage view of the **Advanced Tiles Setup**.

Tile Region List Options

Parameter	Description
Tile Regions List	Displays the added tile regions. The list contains the following columns:
- Checkbox	Activates the relevant list entry for acquisition.
- Name	Here you can edit the name of the tile region.
- Category	Displays the category of the tile region. Categories can be defined in the view options of the advanced tiles setup on the properties tab.
- Tiles	Displays the number of tiles of the tile region.
- Z	Displays the Z-position of the tile region.
- Size	Displays the size of the tile region along its x and y axes in micrometers.
Up and **Down**	With the Up/Down buttons you can shift the selected list entry one position up or down in the tile regions list. This allows you to modify the acquisition order. Note that the order in the list will only be respected if the sorting of tile regions/ positions is deactivated in the **Options** section (**Stage Travel Optimization**)!
Delete	Deletes the selected list entry.
Options	If you click on the button, you see the following options:
- Set Current Z for Selected Tile Regions	Sets the current Z-Position for all selected tile regions.
- Set Current X/Y/Z for Selected Tile Regions	Sets the current X/Y/Z-Position for all selected tile regions.
- Delete	Deletes the current tile region.
- Delete All	Deletes all tile regions.
- Activate	Activates the current tile region for acquisition.

Parameter	Description
- Deactivate	Deactivates the current tile region for acquisition.
- Unlock	Unlocks the current tile region. The tile regions or positions are only locked if created in carrier mode.
- Unlock All	Unlocks all locked tile region.
- Sort	**By Center Position (Y -> X)** sorts all tile regions according to their overall Y position. **By Center Position (X -> Y)** sorts all tile regions according to their overall X position. **By Category** sorts all tile regions according to their category. Note that the order in the list will only be respected if the sorting of tile regions/ positions is deactivated in the **Options** section (**Stage Travel Optimization**)!
- Convert to Positions…	Converts a selected tile region into Positions or a Position Array.
Properties Tile Region	Only visible if you have the **Tiles & Positions** license. Displays the name of the currently selected tile region if one region is selected.
Category	Only visible if you have the **Tiles & Positions** license. Shows the currently assigned category of the selected tile region. The **Default** category is set for all new tile regions.
Options	Only visible if you have the **Tiles & Positions** license. Opens the options for editing and creating categories.
- New	Opens the **New Category** dialog to create a new category.
- Edit	Opens the **Edit Category** dialog to edit the selected category.
- Delete	Deletes the selected category and sets the category of the tile region to **Default**.
X	Only visible if you have the **Tiles & Positions** license. Displays and sets the x-value of the selected tile region.
Y	Only visible if you have the **Tiles & Positions** license. Displays and sets the y-value of the selected tile region.
Z	Only visible if you have the **Tiles & Positions** license. Displays and sets the z-value of the selected tile region.
Set Current Z	Only visible if you have the **Tiles & Positions** license. Sets the **Z** dimension at the current **Z** position of the focus drive.
Width	Only visible if you have the **Tiles & Positions** license. Displays and sets the width of the selected tile region.
Height	Only visible if you have the **Tiles & Positions** license. Displays and sets the height of the selected tile region.
Verify	Only visible if you have the **Tiles & Positions** license.

Parameter	Description
	Opens the **Verify Tile Regions** dialog. There you can verify each point of the tile region according focus und position.

12.19.14.1.2 Positions Section

Current Position section

Displays the current stage position.
Note: This section is only visible if you have no license for **Tiles & Positions**. With a license, this functionality is visible in the *Left Toolbar [▸ 547]* of the **Tiles Advanced Setup**.

Parameter	Description
X Position	Displays the x coordinate of the current position.
Y Position	Displays the y coordinate of the current position.
Add	Adds the current position to the **Positions List** and activates it for acquisition.

Display mode section

Parameter	Description
Single Positions	Shows the **Single Positions List**. To learn more about single positions see glossary "Position".
Position Arrays	Shows the **Position Arrays List** and the **Positions of selected Array List**, that shows a full **Single Positions List** for the selected position array. To learn more about position arrays see glossary "Position".
Single Position List / Position Array List	Displays the added positions/ position arrays. The list contains the following columns and buttons.
– Checkbox	Activates the relevant list entry for acquisition.
– Name	Displays and edits the name of the single position.
– Category	Displays the category of the single position. Categories can be defined in the view options of the advanced tiles setup on the properties tab.
– Contour	Only visible if you have selected **Position Arrays**. Displays the contour of the position array.
– Positions	Only visible if you have selected **Position Arrays**. Displays the number of positions of the position array.
– Size	Only visible if you have selected **Position Arrays**. Displays the size of the position array.
– X	Displays the x-position of the single position.
– Y	Displays the y-position of the single position.

Parameter	Description
– Z	Displays and edits the z-position of the single position.
– and	With the buttons you can shift selected list entry one position up or down in the tile regions list. This allows you to modify the acquisition order. Note that the **Tile Regions/Positions** checkbox has to be deactivated in *Tiles Options [▸ 555]*. Note that the order in the list will only be respected if the sorting of tile regions/ positions is deactivated in the **Options** section (**Stage Travel Optimization**)!
– Delete	Deletes the selected list entry.
– Options	Opens the *Options for editing Single Positions [▸ 537]* or *Options for editing Position Arrays [▸ 537]* respectively.
Verify	Opens the *Verify Tile Regions or Verify Positions Dialog [▸ 542]*.
Properties Position	Only visible if you have the **Tiles & Positions** license. Displays the name of the currently selected position.
Category	Only visible if you have the **Tiles & Positions** license. Shows the currently assigned category of the selected tile region. The **Default** category is set for all new tile regions.
Options	Only visible if you have the **Tiles & Positions** license. Opens the options for editing and creating categories.
- New...	Opens the **New Category** dialog to create a new category.
- Edit…	Opens the **Edit Category** dialog to edit the selected category.
- Delete	Deletes the selected category and sets the category of the tile region to **Default**.
X	Only visible if you have the **Tiles & Positions** license. Displays and sets the x-value of the selected tile region.
Y	Only visible if you have the **Tiles & Positions** license. Displays and sets the y-value of the selected tile region.
Z	Only visible if you have the **Tiles & Positions** license. Displays and sets the z-value of the selected tile region.
Set Current Z	Only visible if you have the **Tiles & Positions** license. Sets the Z dimension at the current Z position of the focus drive.

12.19.14.1.2.1 Options for editing Single Positions

Parameter	Description
Set Current Z for Selected Positions	Sets the current Z-Position for all selected positions.
Set Current XYZ for Selected Position	Sets the current X-Y-Z-Position for the selected position.
Delete	Deletes the current position.
Delete All	Deletes all positions.
Activate	Activates the current position for acquisition.
Deactivate	Deactivates the current position for acquisition.
Sort	Sorts the list entries according to the chosen parameter.
- By Center Position (Y -> X)	Sorts all positions according to their overall Y position.
- By Center Position (X -> Y)	Sorts all positions according to their overall X position.
- By Category	Sorts all positions according to their category.
Import stage marks as positions	Only visible if you have the **Tiles & Positions** license. Imports the marks from the **Stage** tool as positions.

12.19.14.1.2.2 Options for editing Position Arrays

Parameter	Description
Set Current Z for all Positions in selected Arrays	Sets the current Z-Position for all positions in the selected arrays.
Delete	Deletes the current position array.
Delete All	Deletes all position arrays.
Activate	Activates the current position array for acquisition.
Deactivate	Deactivates the current position array for acquisition.
Unlock	Unlocks the current position array.
Unlock All	Unlocks all locked position arrays.
Sort	Sorts the list entries according to the chosen parameter.
- By Center Position (Y -> X)	Sorts all positions according to their overall Y position.
- By Center Position (X -> Y)	Sorts all positions according to their overall X position.

12.19.14.1.3 Sample Carrier Section

Only visible if the **Show All** mode is activated and only available with a licence for the **Tiles & Position** module.

Parameter	Description
Sample Carrier	Displays the selected sample carrier template. If no template is selected it will display **None**.
Select...	Opens the *Select Template Dialog [▸ 557]*. Here you can select the sample carrier template.
(Edit Support Points)	Opens the sample carrier selection/editor dialog. Here you can edit and add global support points to the selected sample carrier.
Delete	Deletes the selected sample carrier from the sample carrier field. The template will still be available in the **Select Sample Carrier Template** dialog.
Calibrate...	Only available if a suitable channel is configured for the experiment. Opens the **Sample Carrier Calibration Wizard** which guides you through the sample carrier calibration.
Move Focus Drive to Load Position Between Containers	**Activated:** Moves the focus drive to the loading position during the movement of the stage to another container of the sample carrier (e.g. a well or slide). This prevents possible damage. Note that this behavior is only applied during an experiment.

12.19.14.1.4 Focus Surface and Support Points Section

Only visible if the **Show All** mode is activated.

Parameter	Description
Selected Tile Region	Displays the number of currently selected tile regions.
Current Position	Adds a support point at the current stage and focus position. Only available if the stage is positioned within the yellow bounding of the selected tile region.
Center of Tile Region	Adds a support point at the center of the currently selected tile region.
Method	Selects which method is used to add multiple support points.
- **Generic**	Distribution method with a simple column and row approach. ZEISS recommends using this method for smaller tile regions (<200 tiles) of a regular shape, e.g. quadratic, rectangular, and circular.
- **Onion Skin**	Distribution method for mid- or larger tile regions (>200 tiles) of an irregular shape like you might use to image large area tissue specimens, e.g. brain slices.
Columns	Only available with **Generic**. Sets the number of columns of support points within the selected tile region.
Rows	Sets the number of rows of support points within the selected tile region.

Parameter	Description
Density	Only available with **Onion Skin**. Determines the number of support points (up to a preset maximum number) that are used to cover the tile region. Example: A square tile region with 400 tiles has 20 support points with the default parameters. The parameter density is set with a standard value of 5%. This means that in a, for example, approximately square tile region of 400 tiles about 20 support points are created.
Margin	Only available with **Onion Skin**. This parameter allows you to control how close support points can be placed along the edges of your tile region. The value defines the distance to the tile regions boundary as a number of tiles. The default is 1 and allows support points to be placed in the outer most "layer" of tiles. A value of 2 allow support points only from 2 "layer" of tiles from the boundary, etc. This feature is useful if a software autofocus (SWAF) is used to determine the z-values of your support points. Sometimes tiles in the boundary regions might not contain any structure. Thus, the SWAF cannot adjust the support points z-value.
Max.	Only available with **Onion Skin**. Determines the maximum number of support points for a tile region. As the density parameter is set to 5%, this would necessitate for very large specimens that the number can get very large. But a larger number of focus points does not always mean a better quality calculation of the focus surface. For this reason, you can define a maximum number of focus points in the range of 24 to 36 points.
Distribute	Distributes the entered number of support points defined in the column and row input fields within the tile region. Previously defined support points will be deleted.
Local (per Tile Region) / Global (on Carrier) tab	Displays a list with local or global support points. You have the following columns and options:
X	Displays and edits the x coordinate of the focus reference point.
Y	Displays and edits the y coordinate of the focus reference point.
Z	Displays and edits the z coordinate of the focus reference point.
Container	Allows you to sort the global support points according to their container on the sample carrier.
+ **Add**	Only visible on the **Global (on Carrier)** tab. Adds a new support point to the selected tile region at the current stage and focus position.
Delete	Deletes the selected list entry.

Parameter	Description
Options	
– Add Support Point at Current Stage and Focus Position	Adds a new support point at the current stage and focus position.
– Set Current Z for Selected Support Points	Sets the current Z-Position for all selected support points.
– Set Current X/Y/Z for Selected Support Points	Sets the current X-Y-Z-Position for the selected support point.
– Delete	Deletes the current support point.
– Delete All	Deletes all support points from the current tile region.
– Delete all Support Points from Selected Tile Regions	Deletes all support points from the selected tile regions.
– Delete all Support Points from all Tile Regions	Deletes all support points from all tile regions.
– Set Current Z for Selected Support Points	Only visible on the **Global (on Carrier)** tab. Sets the current z-position for all selected support points.
Set current XYZ	Sets the current x/y/z position for the selected support point.
Set current Z	Sets the current z-position for all selected support points.
Interpolation Degree	Displays the selected degree of interpolation. Selects a degree of interpolation from the dropdown list.
Verify	Opens the dialog to verify the support points.

The more variable the surface of your specimen the higher you should choose the interpolation degree. For higher degrees you will need more support points. The minimum number of support points required for each interpolation degree is given in the dropdown list. As an overachievement of this minimum number ensures a solid calculation, we recommend minimizing the interpolation degree even if you added more support points. Increase the interpolation degree only so far as the surface condition of your specimen demands. If the number of support points is too low for the selected interpolation degree, the next lower level for which the minimum is forefilled will be used. By default, ZEN uses the second order parabolic saddle surface that requires at least 9 support points. For most applications you will not need to adjust this setting.

The properties of a selected global support point slightly differ from those of a local one as you cannot edit the **X/Y** dimensions because they are fixed by the sample carrier template you have selected. Therefore there is no **Set Current X/Y/Z** button for global support points. If you want to edit the number and XY dimension of your global support points this can be done directly via the **Sample Carrier** section of the **Tiles** tool.

12.19.14.1.5 Options Section

Only visible if the **Show All** mode is activated.

Here you can set options like acquisition and stage travel behavior during the experiment. Changes in this section of the tool affect all elements, tile acquisitions, positions and position arrays.

Parameter	Description
Tile Overlap	Defines the overlap in percent of individual tiles of the tile regions. The value is set to 10 % by default. Note that lower overlap might cause artifacts when stitching the image as there is less information for a robust correlation. No overlap will not allow the images to be stitched correctly.
Stage Travel Optimization	In this section you can adjust settings for stage traveling during an experiment or preview scan (only with **Tiles & Positions** module). Note that in some cases the preview scan function will automatically select a travel mode for the stage that is more appropriate.
Travel in Tile Regions	
- Meander	Acquires tile regions following a meander pattern – alternately from both travel directions (left -> right; right -> left). This scan movement is faster.
- Comb	Acquires tile regions following a comb pattern – always from one travel direction only (left -> right). This scan movement is more precise.
- Spiral	Acquires tile regions following a spiral pattern – from the center of the region to the outer bounds in a clockwise motion. This mode works only for regions with rectangular or elliptical contours.
Tile Regions/Positions	**Activated**: Individual positions and tile regions are not acquired in the sequence in which they are defined in the **Tile Regions** list. The stage movement will be automatically adapted to the location of the individual tile regions and positions. If you add or remove tile regions or positions, the sequence of acquisition therefore also changes.
- Sort by X, then Y	The tile regions and positions are sorted by their absolute position (first x, then y).
- Sort by Y, then X	The tile regions and positions are sorted by their absolute position (first y, then x).
Carrier Wells/Container	**Activated**: Applies the selected travel patterns (meander or comb) when acquiring tiles in sample carriers with wells/containers. When, for example meander is used, the stage travel between wells will be e.g. A1, A2 ....A4 --> B4, B3 .....B1 etc.

Parameter	Description
- Meander	Acquires tile regions following a meander pattern – alternately from both travel directions (left -> right; right -> left). This scan movement is faster.
- Comb	Acquires tile regions following a comb pattern – always from one travel direction only (left -> right). This scan movement is more precise.
Use Stage Speed from Stage Tool	This function is only visible if the corresponding option is activated in **Tools > Options > Acquisition > Tiles & Positions**. By default, backlash correction will be used during stage movement. **Activated:** The software uses the stage travel speed which is set in the **Stage** tool (Right Tool Area).
Stage and Focus Backlash Correction	**Activated**: Stage and focus positioning is done with a backlash correction which is more precise but slightly slower.
Move Focus to Load Position Between Regions/Positions	**Activated**: The focus drive is moved to the load position while moving to another tile region, position, or well.
Split Scenes into Separate Files	**Activated**: The scenes (e.g. tile regions and positions) are stored into separate physical files. They are still combined into one logical image file.
Image Pyramid During Acquisition	**Activated**: An image pyramid is generated during the acquisition. This optimizes the image for fast display. If this option is not activated, the acquired image will not be shown and updated in the document area while the acquisition is running.

12.19.14.1.6 Verify Tile Regions or Verify Positions Dialog

Note that the z values of positions, tile regions (unless they have at least one support point), local support points, and global support points are verified in a separate dialog accessed via the **Focus Surface and Support Points** section. As the dialog contains the same items and options for verifying the Z values it is described here once.

Parameter	Description
Tile Regions/Positions List	Displays all the tile regions (TR), positions (P), or support points (SP) currently active in your experiment. The list contains the following columns and buttons:
- Status	Here you can see if the Z-Position is already verified. Then a green checkmark will appear in the corresponding row.
- Name	Displays the name of the selected object.
- Z (µm)	Displays the z-position of the tile region, position, or support point.
- Tile Region	Shows the tile region to which the local support point belongs.
- Array	Shows if the position is part of an Position Array.

Parameter	Description
- Options	Opens the options menu for verifying tile regions / positions, see description below.
Verification Helper Method	
- None (manual adjustment)	If selected, you need to manually adjust the focus for each point to be verified.
- Autofocus (AF)	Only available if your system has a motorized focus drive (z-axis) and the software autofocus module. If selected, you can use the software autofocus for adjusting the focus for each point to be verified. The corresponding buttons will then appear in the dialog.
- Definite Focus (AF)	Only available if your system has a **Definite Focus** device. If selected, you can use the definite focus for adjusting the focus for each point to be verified. The corresponding buttons will then appear in the dialog further below.
Move to Current Point	Moves the stage to the selected object.
Include Z when Moving to Points	**Activated:** ZEN moves to the selected object and adjusts the position of the z-drive to the currently assigned z-value.
Set Z & Move to Next	Sets the current z value for the selected support point and sets the status to verified. Then the software moves the stage to the next support point.
Run AF (or DF) and Set Z	This button is only visible if you have selected **Autofocus (AF)** or **Definite Focus (DF)** as Helper Method. Runs the software autofocus / definite focus and sets the current z position to verified.
Use AF (or DF) to Verify the Remaining	This button is only visible if you have selected **Autofocus (AF)** or **Definite Focus (DF)** as Helper Method. Automatically moves to the remaining points and determines the z value with the software autofocus for them.

Options for verifying tile regions/positions

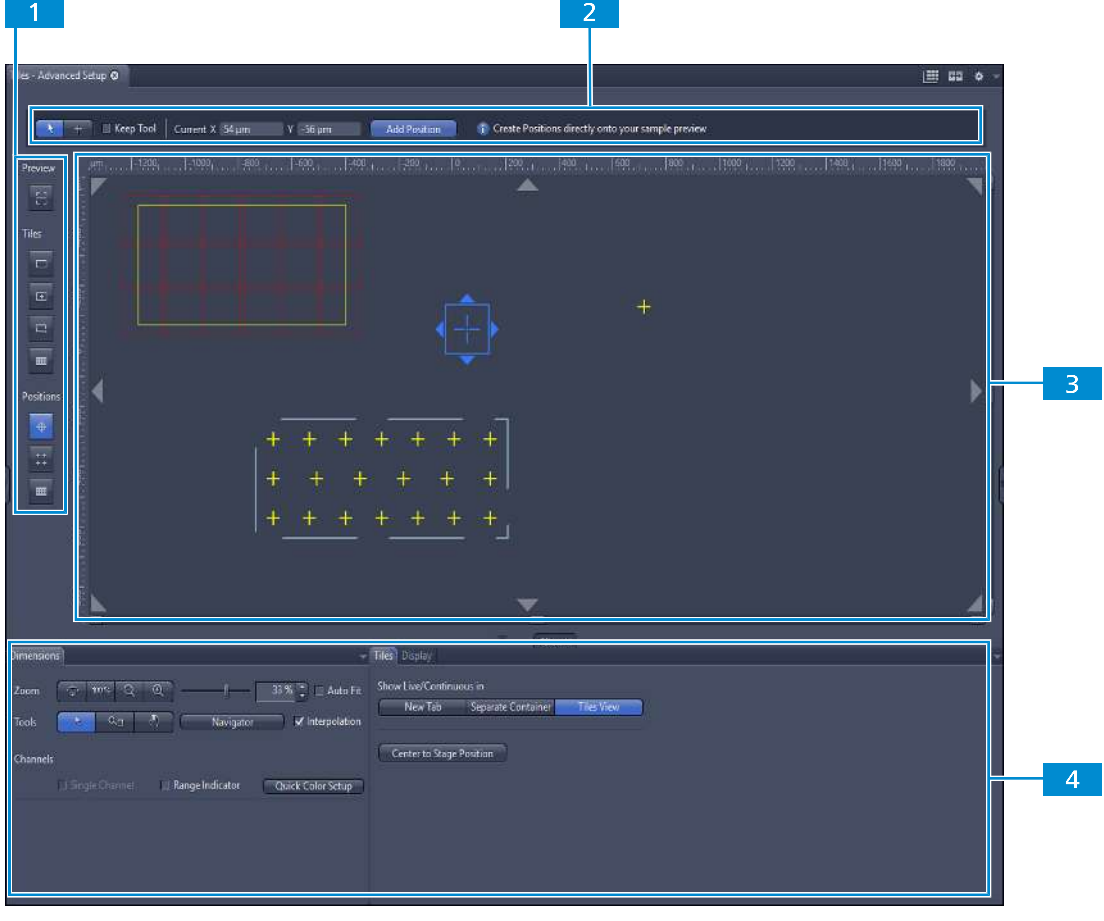
Parameter	Description
Current Point Verified	Here you can change the status of the selected support point from verified to unverified (or vice versa).
Set all Points as Verified	Changes the status of all points to verified.
Reset Verification State of all Points	Changes the status of all points to unverified.

Parameter	Description
Set Current Z for Selected Points	The current z value is set for all selected points. You can press *Ctrl* and the left mouse button to select multiple points, or *Ctrl + A* to select all.
Set Current Z for all Points	The current z value is set for all points.
Apply Z-Offset...	Opens al dialog to apply a z-offset for all or the selected points.

12.19.14.2 Tiles Advanced Setup

Here you can visualize and plan your Tiles and Positions experiment. The advanced tiles setup opens by clicking on the **Show Viewer** button in the **Tiles** tool on the **Acquisition** tab of the **Left Tool Area**. In the **Center Screen Area** you can see the *Stage view [**545]*. When the **Tiles Advanced Setup** opens, the stage view is zoomed to a predefined factor. You can change the **Zoom** in the **Dimensions** tab, or by scrolling the mouse wheel.
To navigate around you have the following options:

- Move the mouse pointer of the live navigator, then press and hold the left mouse button and drag the live navigator to the desired location. Release the left mouse button to finish.
- Place the mouse pointer at a location where you want to move the stage and double click.
- Use the software joystick in the **Stage** tool of the **Right Tool Area** to navigate. Alternatively use the hardware joystick of your stage controller.
- In each corner and along each edge with the arrowheads, you can click to move the view in this direction. Additional settings and tools relating to tile regions or positions can be found in the specific view options.



1 **Left Toolbar**
Here you select which setup you use to set tile regions or position. for more information, see *Left Toolbar [▸ 547]*.

2 **Top Toolbar**
Depending on what you have selected on the **Left Toolbar**, different features to create tiles or positions are displayed here. For more information, see *Top Toolbar [▸ 547]*.

3 **Stage View**
The **Stage View** shows the full travel range of the microscope stage, along with the current stage position, the graphical display of sample carriers, and your acquired mosaic images. For more information, see *Stage View [▸ 545]*.

4 **View Options**
General and *tiles specific [▸ 554]* view options are displayed here.
Note that the functionalities between activated and deactivated **Show All** layout might differ.

12.19.14.3 Stage View

The **Center Screen Area** shows the full travel range of the microscope stage, along with the current stage position, the graphical display of sample carriers and your acquired mosaic images. You can control the stage view using the arrow icons at the edges of the image area. The view can be enlarged, reduced or moved using the general control elements.

Symbol	Parameter	Description
/	**Selected/Active**	Represents the **Selected/Active** container/well by a blue border.
	Live Navigator	Shows the current stage position including the live image as a frame outlined in blue. To move the frame, double-click on the position to which you want to move it. Alternatively hold the left mouse button on the live navigator tool while dragging the mouse. The frame can also be used to control acquisition. If you click on one of the frame's blue arrow icons, an image is acquired. The Live Navigator tool is moved one frame width in the relevant direction. You can create tile images of your sample easily in this way.
	Tile Region	Represents the tile regions in the stage view by a red grid.
	Positions	Represents positions in the stage view by a yellow plus symbol wit a red frame.
	Position Array	Represents the position arrays in the stage view by the corresponding position symbols surrounded by a dashed line.

Symbol	Parameter	Description
	Local Support Point	Represents local support points in the stage view by a yellow circle with a dot in the middle.
	Global Support Point	Represents global support points in the stage view by a white circle with a dot in the middle.

A right-click in the **Stage View** displays a context menu with *Tiles & Positions specific options* [▸ 546].

12.19.14.3.1 Specific Tiles & Positions Context Menu Options

When you right click in the **Stage View** of the **Tiles Advanced Setup**, you have the following **Tiles & Positions** specific options:

Parameter	Description
Show Images	**Activated:** Displays images in the **Stage View**.
Remove Images	Displays the options of what images are removed from the **Stage View**.
– All	Removes all images.
– All Manual Snap Images	Removes all manually taken snap images.
– All Preview Scan Images	Removes all preview scan images including preview images imported into the **Stage View**.
Import Preview Image	Opens the file browser to select an image which gets imported into the **Stage View**.
Save Preview Images	Opens the preview images in separate image documents in ZEN, which allows you to save them or work with them if necessary.
Bring Navigator into View	Re-centers the **Stage View** onto the current stage position coordinates at the preset zoom level. You can also activate this function with *Ctrl + B*.
Show Tile Regions Name	**Activated:** Displays the names of the of the tile regions in the **Stage View**.
Show Tile Rectangle for Positions	**Activated:** Displays a red rectangle around the individual positions in the **Stage View**.

12.19.14.4 Tiles Toolbars

In the **Tiles - Advanced Setup** your main features to create regions and positions are in two toolbars on top and on the left of the viewer. The options available in the top toolbar depend on what is selected in the left toolbar.

12.19.14.4.1 Left Toolbar

Here you select which setup you use to set tile regions or positions.

Parameter	Description
Preview	Displays the tools to define the settings for a preview scan (see also *Preview Scan Toolbar [▸ 548]*). Typically a low magnification objective and a channel which protects your sample (e.g. transmitted light) is used. This will give you a low resolution overview of the sample to mark tile regions and/or positions.
Tiles	Here you can select which setup you want to use to set the tile regions. The corresponding tools are displayed in the *Top Toolbar [▸ 547]*.
– Setup by contour	Displays the tools to define tile regions by means of contour (see also *Contour Toolbar [▸ 548]*).
– Setup by predefined	Displays the tools to define tile regions by means of number or size (see also *Predefined Toolbar [▸ 549]*).
– Setup by stakes	Displays the tools to define tile regions by specifying two or more marker positions (see also *Stake Toolbar [▸ 550]*).
– Setup by carrier	Displays the tools to define tile regions automatically by means of the fill factor of the sample carrier (see also *Tiles Carrier Toolbar [▸ 550]*).
Positions	Here you can select which setup you want to be used to set the positions. The corresponding tools are displayed in the *Top Toolbar [▸ 547]*.
– Setup by location	Displays the tools to define positions by means of the location (see also *Positions Location Toolbar [▸ 551]*). You can add various positions in the Stage View using the mouse.
– Setup by array	Displays the tools to define positions by means of position arrays (see also *Position Array Toolbar [▸ 551]*). You can add various contours for position arrays in the stage view.
– Setup by carrier	Displays the tools to define positions automatically on the relevant sample carrier (see also *Positions Carrier Toolbar [▸ 552]*).

12.19.14.4.2 Top Toolbar

Depending on what you have selected on the *Left Toolbar [▸ 547]* of the **Tiles - Advanced Setup**, different features to create tiles or positions are displayed. For information on the various top toolbars, see the list below.

12.19.14.4.2.1 Preview Scan Toolbar

Here you can define the settings for a preview scan. Typically a low magnification objective and a channel which protects your sample (e.g. transmitted light) is used. This will give you a low resolution overview of the sample to mark tile regions and/or positions.

Parameter	Description
Use Experiment Settings	Uses the settings of the experiment for the preview scan.
Channel	Only available if **Use Experiment Settings** is deactivated. Displays the channels for the preview scan. If you deactivate **Use Experiment Settings**, you can deselect individual channels here.
Camera Binning	Only available if **Use Experiment Settings** is deactivated. Sets the binning of the camera for the preview scan experiment only. The exposure time will be compensated automatically for the preview scan. Note that higher binning will reduce the scan time (due to shorter exposures of the camera), but reduce the image resolution.
Delete existing preview images	**Activated:** Deletes the already existing preview images before the preview scan is started.
Start	Starts the preview scan to acquire the overview image.

See also

Generating a Preview Scan [▸ 515]

12.19.14.4.2.2 Contour Toolbar

Here you can define the tile regions by means of the contour.

Parameter	Description
Selection Mode	With this tool you can select an already created tile region by clicking on it to move or edit it.
Rectangle	With this tool you can draw a rectangle tile region.
Ellipse	With this tool you can draw a elliptical tile region.
Polygon	With this tool you can draw a polygonal tile region.
Keep Tool	**Activated:** Keeps the selected tool active. You can use the tool several times in succession without having to reselect it.

See also

Creating Tile Regions by Contour [▸ 517]

12.19.14.4.2.3 Predefined Toolbar

Here you can define the tile regions by means of the number or size.

Parameter	Description
Rectangle	Draws a rectangular tile region.
Circle	Draws a circular tile region
Tiles	Using this mode you have to enter the number of tiles as a reference for the size of the tile region. Enter the number of tiles in the **X / Y** input fields. If you are adding a circular tile region, enter the number of tiles for the diameter in the **Diameter** input field.
Size	Using this mode you have to enter the size as a reference for the size of the tile region. Enter the size of the tile region in the **X / Y** input fields. If you are adding a circular tile region, enter the diameter of the tile region in the **Diameter** input field.
Top left anchor position	The anchor of the defined shape is at the top left.
Center anchor position	The anchor of the defined shape is centered.
Bottom right anchor position	The anchor of the defined shape is at the bottom right.
Add Tile Region	Adds the tile region to the **Tile Regions List** and activates it for acquisition. Added tile regions are displayed in the form of red grids in the stage view of the **Advanced Tiles Setup**.
Selection	Select an element in the stage view to edit or move it.
Add Tile Region	Adds the current tile definition in the image area.
Keep Tool	**Activated:** Keeps the selected tool active. You can use the tool several times in succession without having to reselect it.

See also

Creating Tile Regions by Predefined [518]

12.19.14.4.2.4 Stake Toolbar

Here you can define the tile regions with stake markers.

Parameter	Description
Add Marker Position	Adds a marker position to the current stage and focus position.
Remove Marker Position	Removes the last marker position.
Rectangle	Draws a rectangular tile region.
Circle	Draws a circular tile region
Polygon	Draws a polygonal tile region
Done	Saves the current tile regions setup and resets the marker positions.

12.19.14.4.2.5 Tiles Carrier Toolbar

Here you can define the tile regions automatically by means of the fill factor of the sample carrier.

Info

- A sample carrier must have been selected in the *Sample Carrier [**▸ 538]* section of the **Tiles** tool.
- Manually created tile regions and positions (setup by **Contour** and setup by **Predefined**) will be deleted, if you switch to the setup by **Carrier**. If you want to combine manual and automatic setup, first use setup by **Carrier** and then switch to a manual setup. Tile regions that are created automatically by setup by **Carrier**, are defined to a container and permanently assigned and locked by default, against manual editing. You can unlock the tile regions in the **Tiles** tool by selecting the desired tile region and unlocking it via the options menu in the tile regions list. You can also unlock all tile regions here if necessary.

Parameter	Description
Select Sample Carrier	Only visible if no sample carrier is currently selected. Opens the dialog to select a sample carrier template.
Create	Creates a tile region for every selected sample carrier container.
Remove	Removes the tile region in every selected sample carrier container.
Fill Factor	Here you can enter the fill factor used to fill the selected container.

Parameter	Description
Columns/Rows	Here you can add single tile regions to a container by defining the number of columns and rows of the tile. The tile region is always placed at the center of the well container.

See also

Creating Tile Regions by Carrier [518]

12.19.14.4.2.6 Positions Location Toolbar

Here, you can define the positions by means of the location. You can add various positions in the **Stage View** using the mouse.

Parameter	Description
Selection	Selects an element in the stage view to edit or move it.
Add	Adds a new position on the stage view.
Keep Tool	**Activated:** Keeps the selected tool active. You can use the tool several times without having to reselect it.
Current X / Y	Displays the current stage position (X/Y).
Add Position	Adds a new position at the current stage position.

See also

Creating Positions by Location [518]

12.19.14.4.2.7 Position Array Toolbar

Here you can define the positions by means of position arrays. You can add various contours for position arrays in the stage view.

Parameter	Description
Selection	With this tool you can select an already created position array to move or edit it.
Rectangle	With this tool you can draw a rectangular position array.
Ellipse	With this tool you can draw a elliptical position array.
Keep Tool	**Activated:** Keeps the selected tool active. You can use the tool several times in succession without having to reselect it.
Distribute Positions by	
– Number	Activates the input for **Number** and **Bias**.

Parameter	Description
– Grid	Activates the input field for an **Overlap**.
Number	Only visible if **Number** is selected. Shows the current number of positions that are distributed to newly created position array. Change the number to increase or decrease the number of single positions obtained by a position array.
Bias	Only visible if **Number** is selected. Adjusts the overall position of the single positions in the position array.
- None	The single positions of the position array will be distributed evenly within the array.
- Center	The single positions of the position array will mainly be distributed near to the center of the position array. Less positions will be at the edges of the array.
- Edge	The positions of the position array will be distributed to the edges of the array. Less positions will be in the center of the array.
Random	Only visible if **Number** is selected. **Activated:** The single positions will be distributed randomly within the position array. The overall bias will still be taken into account.
Overlap	Only visible if **Grid** is selected. Shows the current degree of overlap in % of each position in the array relative to its neighbors. Both positive and negative values are possible.

See also

Creating Positions by Array [519]

12.19.14.4.2.8 Positions Carrier Toolbar

Here you can define the positions automatically on the relevant sample carrier.

Info

- A sample carrier must have been selected in the *Sample Carrier [538]* section of the **Tiles** tool.
- Manually created tile regions and positions (setup by **Contour** and setup by **Predefined**) will be deleted, if you switch to the setup by **Carrier**. If you want to combine manual and automatic setup, first use setup by **Carrier** and then switch to a manual setup. Tile regions that are created automatically by setup by **Carrier**, are defined to a container and permanently assigned and locked by default, against manual editing. You can unlock the tile regions in the **Tiles** tool by selecting the desired tile region and unlocking it via the options menu in the tile regions list. You can also unlock all tile regions here if necessary.

Parameter	Description
Select Sample Carrier	Only visible if no sample carrier is currently selected. Opens the dialog to select a sample carrier template.

Parameter	Description
+ **Create**	Creates a position array for every selected container of the sample carrier.
– **Remove**	Removes the position array for every selected container of the sample carrier.
Distribute Positions by	
– Number	Activates the input for **Number** and **Bias**.
– Grid	Activates the input field for an **Overlap**.
Number	Only visible if **Number** is selected. Shows the current number of positions that are distributed to newly created position array. Change the number to increase or decrease the number of single positions obtained by a position array.
Bias	Only visible if **Number** is selected. Adjusts the overall position of the single positions in the position array.
- None	The single positions will be distributed evenly within the array.
- Center	The single positions will mainly be distributed near to the center of the position array. Less positions will be at the edges of the array.
- Edge	The positions will be distributed to the edges of the array. Less positions will be in the center of the array.
Random	Only visible if **Number** is selected. **Activated:** The single positions will be distributed randomly within the position array. The overall bias will still be taken into account.
Columns	Only visible if **Grid** is selected. Sets the number of columns of the position array in each container of the sample carrier.
Rows	Only visible if **Grid** is selected. Sets the number of rows of the position array in each container of the sample carrier.
Overlap	Only visible if **Grid** is selected. Shows the current degree of overlap in % of each position in the array relative to its neighbors. Both positive and negative values are possible.

See also

Creating Positions by Carrier [519]

12.19.14.5 Tiles View Options

This view options are specific for the **Tiles & Positions** module.

12.19.14.5.1 Tiles Tab

Here you can define how the software behaves when the live image is started and the **Tiles - Advanced Setup** is open.

Parameter	Description
New Tab	Opens the live or continuous image into a new image container.
Separate Container	Opens a live or continuous image into a separate container. Both the **Tiles - Advanced Setup** and the live or continuous images are visible in parallel. We recommend to activate this checkbox, especially if you use two separate monitors. You may also want to use the Automatic Contanier Layout setting found in the view option dropdown list of the *Document bar [▸ 22]* in the *Center Screen Area [▸ 21]*.
Tiles View	Displays the live or continuous image in place in the **Stage View** of the **Tiles - Advanced Setup**.
Center to Stage Position	You can use this function if you need quickly to re-find your current location. Re-centers the **Stage View** onto the current stage position coordinates at the preset zoom level. You can also activate this function with *Ctrl* + *B*. You can call the function to focus the image with the mouse wheel by pressing and holding the *Ctrl* key when the mouse cursor is over the stage view.

12.19.14.5.2 Carrier Tab

Only visible if a sample carrier is selected.
Here you can see a graphical preview of the sample carrier being used.

Info

Only the containers/wells whose tile regions and positions were set up with the *Tiles Carrier Toolbar [▸ 550]* or the *Positions Carrier Toolbar [▸ 552]* will be taken into account.

Please note the following features of the display:

Symbol	Description
/	**Empty** containers/wells, meaning that no tile regions or positions were set up with the Carrier option, are represented by a grey container/well.
/	The currently **Active** container/well is represented by a blue border.
/	A container/well only filled with **Tile Regions** is represented by a yellow filled container/well.
/	A container/well only filled with **Positions** is represented by a orange filled container/well.
/	A container/well filled with **Tile Regions** and **Positions** is represented by a half yellow, half orange filled container/well.

Info

Right click opens a small context menu. Here you can copy the contents of the selected well, or paste the contents to the selected or all wells.

12.19.14.6 Tiles Options

The additional options for the tiles module allow to set up several options for image acquisition and additional information. The tiles options dialog can be found in the menu bar under **Tools > Options > Acquisition > Tiles & Positions**.
Note: For the changes to be effective you might need to close and reopen the advanced setup viewer.

Parameter	Description
Automatically Start Live Mode in the Advanced Setup View	**Activated:** Automatically starts the **Live** mode in the **Center Screen Area** if you klick in the **Acquisition** tab in the **Tiles** tool on the **Advanced Setup** button. Uncheck this option to prevent unnecessary specimen bleaching. The default is not activated.
Automatic Snap by Clicking the Live Navigator Buttons	**Activated:** Acquires an image if you click on one of the frame's blue arrow icons. The **Live Navigator** tool moves one frame width in the relevant direction. You can create tile images of your sample easily in this way.

Parameter	Description
Enable Stage Movement with Live Navigator	In the Live navigator tool the current stage position including the live image is shown as a frame outlined in blue. To move the frame, double-click on the position to which you want to move it. Alternatively, place the mouse cursor over the blue frame, press and hold the left mouse button and drag the live navigator to the desired location. **Activated:** Allows you to move the **Live Navigator** tool by dragging it to a new location.
Show Stage and Focus Backlash Correction Setting in the Options	**Activated:** In the Tiles option, the setting to switch the backlash correction on or off is shown. Per default it is hidden.
Delimiter for CSV Export/Import	Specifies the delimiter for a CSV export or import. Select **Comma** (default), **Semicolon** or **Tab**.
Ask Whether Support Points/Positions Should be Overwritten	When the support points and/or positions are determined by a software autofocus run the existing points can be overwritten with the new **Z** values. **Activated:** Shows a message box asking if the points should be overwritten if there is a autofocus **Z** value.
Focus Surface Outlier Determination	Ignores support points that are significantly outside the interpolated focus surface. You have the following setting options available:
- Maximum Interpolation Degree for Outlier Detection	This value can be 0 or 1. If 1 then a linear fit is used to detect the outlier support points. This is the default. If 0 a simple average value is used to detect outliers.
- Threshold in Terms of the Standard Deviation (Sigma)	This parameter defines a threshold value to determine which of the support points are outliers from the fitting process. This is defined by the standard deviation (sigma value) set in the spin box. Support points not meeting this criteria are subsequently ignored when the focus surface is determined.
Delay Time After Stage Movements	Defines a delay period which is used for all stage movements in a tiles and position experiment or movements controlled in the advanced tile setup. The delay helps prevent movement in samples where, for example, a large volume of liquid is present in the sample holder. It can be used with the stage speed and acceleration options to optimise experiments with this type of sample.
Binning Compensation of Exposure Time in Preview Scans	Defines the power to which the binning ratio is modified to automatically determine the exposure time value used for a preview scan were the binning setting between the experiment and preview scan differs. The default value is 2.0 i.e. quadratic. Thus, for example the exposure time would be reduced by a factor of four if the experiment binning is 1x1 and the preview scan binning is 2x2. The value can be varied between 1.0 and 2.0 in steps of 0.1.

Parameter	Description
Live Image in Sample Carrier Calibration Wizard (relevant only for systems with camera)	
- Use Imaging Device from Selected Channel with "Acquisition" Settings	**Activated:** Default setting for the live image that allows navigation and focus interaction during the carrier calibration wizard.
- Use Active Camera with "Locate" Settings	This option is only relevant for systems with a wide field (camera based) detector. **Activated:** Allows you to alternatively apply locate camera settings for use in the carrier calibration wizard (live image). By default the experiment settings for the currently selected channel/track will be used.

12.19.14.7 Select Template Dialog

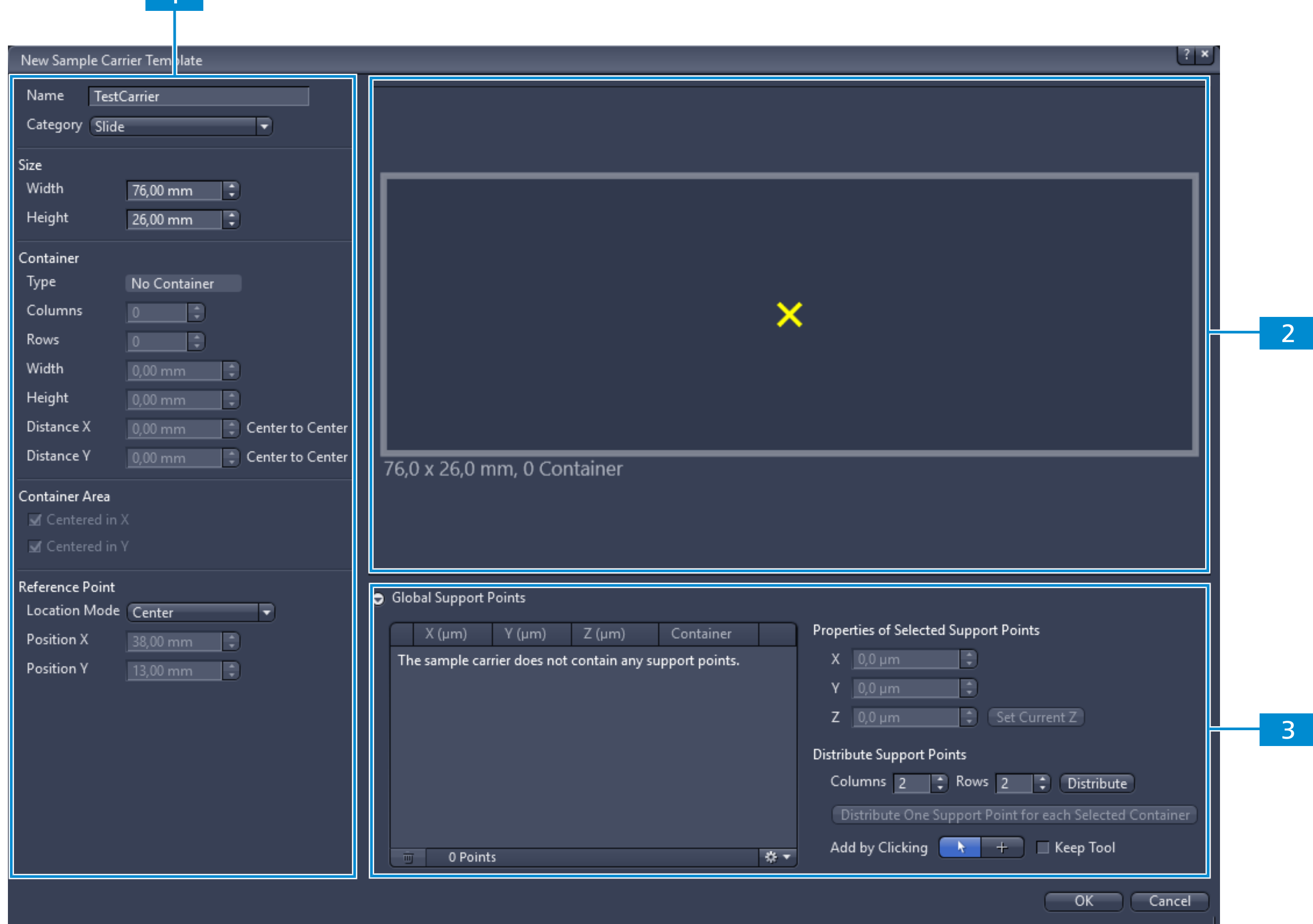
In this dialog you can select a sample carrier or enter a dialog to create a custom carrier. A preview of the selected carrier is shown on the right side of the list.

Parameter	Description
Workgroup Templates	Shows a list of all custom sample carrier templates.
ZEISS Templates	Shows a list of predefined ZEISS sample carrier templates for several sample carriers.
Add	Opens the *New Sample Carrier Template dialog [▸ 558]* to create a new template.
Delete	Deletes the selected sample carrier template.
Options	
– **New Template...**	Opens the *New Sample Carrier Template dialog [▸ 558]* to create a new template.
– **Show/Edit...**	Opens the selected template in the *Edit Sample Carrier Template dialog [▸ 558]* and allows editing. ZEISS templates are read only. If you want to edit a ZEISS template you have to use the **Copy and Edit...** option.
– **Copy**	Copies the selected template.
– **Copy and Edit...**	Copies the selected template and opens it in the *Edit Sample Carrier Template dialog [▸ 558]* dialog for editing.
– **Import**	Imports a template.
– **Export**	Exports the selected template.

Parameter	Description
– **Delete**	Deletes the selected template.
– **Refresh Templates**	Refreshes the list of templates after creating a new one.
OK	Opens the selected sample carrier and closes the dialog.
Cancel	Closes the dialog without opening a sample carrier.

12.19.14.8 Edit Sample Carrier Template dialog

The dialog for editing a template and creating a new one are the same. If you create a new template the dialog fields are empty. On the right you see a live template preview of the setting for the template and global support points.



1 **Basic Template Settings**
Here you have the basic settings for the sample carrier templates. For more information, see *Basic Template Settings [▸ 559]*.

2 **Preview**
Here you can see a preview of the sample carrier.

3 **Global Support Points**
Here you have the settings for global support points. For more information, see *Global Support Points Settings [▸ 560]*.

12.19.14.8.1 Basic Template Settings

Parameter	Description
Name	Shows the name of your template. You can enter/ edit the name.
Category	Shows which sample carrier category the template uses. You can choose between **Slide**, **Multislide**, **Petri Dish**, **Multiwell**, **Multi-chamber** and **Custom**. The category defines the overall appearance of the template and affects the further editing possibilities of the template.
Size	
– Width	Sets the width of the sample carrier template.
– Height	Sets the height of the sample carrier template.
Container	Depending on the category of the template you have different options for editing in this section.
– Type	Shows if the containers of the template are rectangles or circles. If the category is **Custom** you can manually set the type of containers.
– Columns	Shows how many columns of containers the template contains. You can not edit this field if the templates category is **Slide** or **Petri Dish**
– Rows	Shows how many rows of containers the template contains. You can not edit this field if the templates category is **Slide** or **Petri Dish**
– Width Height	Only available for rectangular containers. Determines the width and height of the containers. Not active for **Slide**.
– Diameter	Only available for circular containers. Determines the diameter of the containers. Not active for **Slide**.
– Distance X	Determines the distance in x direction between the containers from center to center. Not active for **Slide** and **Petri Dish**.
– Distance Y	Determines the distance in x direction between the containers from center to center. Not active for **Slide** and **Petri Dish**.
Area	
– Centered in X	**Activated**: The containers will be positioned centered in X direction on the sample carrier template.
– Centered in Y	**Activated**: The containers will be positioned centered in Y direction on the sample carrier template.
Reference Point	The reference point is marked by a **Yellow X** in the preview.
– Location Mode	Defines the position of the templates reference point. You can choose between **Center**, **Top Left**, **Top Right**, **Bottom Left**, **Bottom Right** and **Custom**. With **Custom** you can modify the position directly in the schematic by dragging the yellow reference point X with the mouse cursor. The default position of the reference point varies with the type of carrier.

Parameter	Description
– Position X	Only active if you have selected **Custom** location mode. Sets a custom x position for the reference point of the template.
– Position Y	Only active if you have selected **Custom** location mode. Sets a custom y position for the reference point of the template.

12.19.14.8.2 Global Support Points Settings

To show the section in full, click on the **arrow** button .

Parameter	Description
Global Support Points list	
– X column	Displays the X coordinate of the support point.
– Y column	Displays the Y coordinate of the support point.
– Z column	Displays the Z coordinate of the support point.
– Container column	Shows the container of the support point.
Delete	Deletes the selected list entry.
Options	Opens the Options for editing global support points.
– Set Current Z for Selected Support Points	Sets the current z-value for the selected support points.
– Delete	Deletes the selected support points.
– Delete All	Deletes all support points.
Properties of Selected Support Points	
– X	Sets the X coordinate of the selected support point.
– Y	Sets the Y coordinate of the selected support point.
– Z	Sets the Z coordinate of the selected support point.
– Set Current Z	Sets the Z dimension to the current z position of the stage.
Distribute Support Points	
– Columns	Sets the number of columns of support points within the template.
– Rows	Sets the number of rows of support points within the template.

Parameter	Description
– Distribute	Distributes the entered number of support points defined in the column and row input fields within the template. Previously defined support points will be deleted.
– Distribute One Support Point for each Selected Container	Sets one support point in the center of the selected containers. Previously defined support points will be deleted.
Add by Clicking	
– Selection	Select a support point to move it. Support points distributed by the **Distribute One Support Point for each Selected Container** button can not be moved.
– Add	Add a new support point on the template preview.
– Keep Tool	**Activated:** Keeps the selected tool active.

12.20 ZEN Connect

This module enables you to work with images from multiple sources: zoom in from the full macroscopic view of your sample down to nanoscale details. The Correlative Workspace (CWS) is the efficient way to analyze and correlate images from multiple sources. You can manage, correct, and align these images in 2D as well as in 3D. It works with images from SEM, FIB-SEM, X-ray, light microscopes and any optical images, e.g., from your digital camera. Its sample-centric workspace lets you build a seamless multimodal, multiscale picture of your sample. Use it to guide further investigations and target additional acquisitions.

The module employs a novel graphical user interface concept that makes it easy to investigate all your samples. Design a workflow tailored precisely to the complexity of your experiment, no matter whether it’s a simple task or a compound experiment. A sophisticated workflow environment guides you all the way from the setup for automated acquisition to post processing and customized exports, and right on through to analysis.

12.20.1 Licensing of ZEN Connect

For working with **ZEN Connect** projects or images, you might need a separate license file.
The basic **ZEN Connect** functionality is available for all versions. Additionally, there are the following licenses for **ZEN Connect**:

Connect

This license includes the following:

- Interactive control of stage movement from the correlative workspace
- Export of merged project view as image
- Movie export as fly-through videos
- Import of third-party microscopy images powered by Bioformats
- SerialEM export

Connect 2D Add-on

This license requires the **Connect** license. Additionally, it includes the following functionalities:

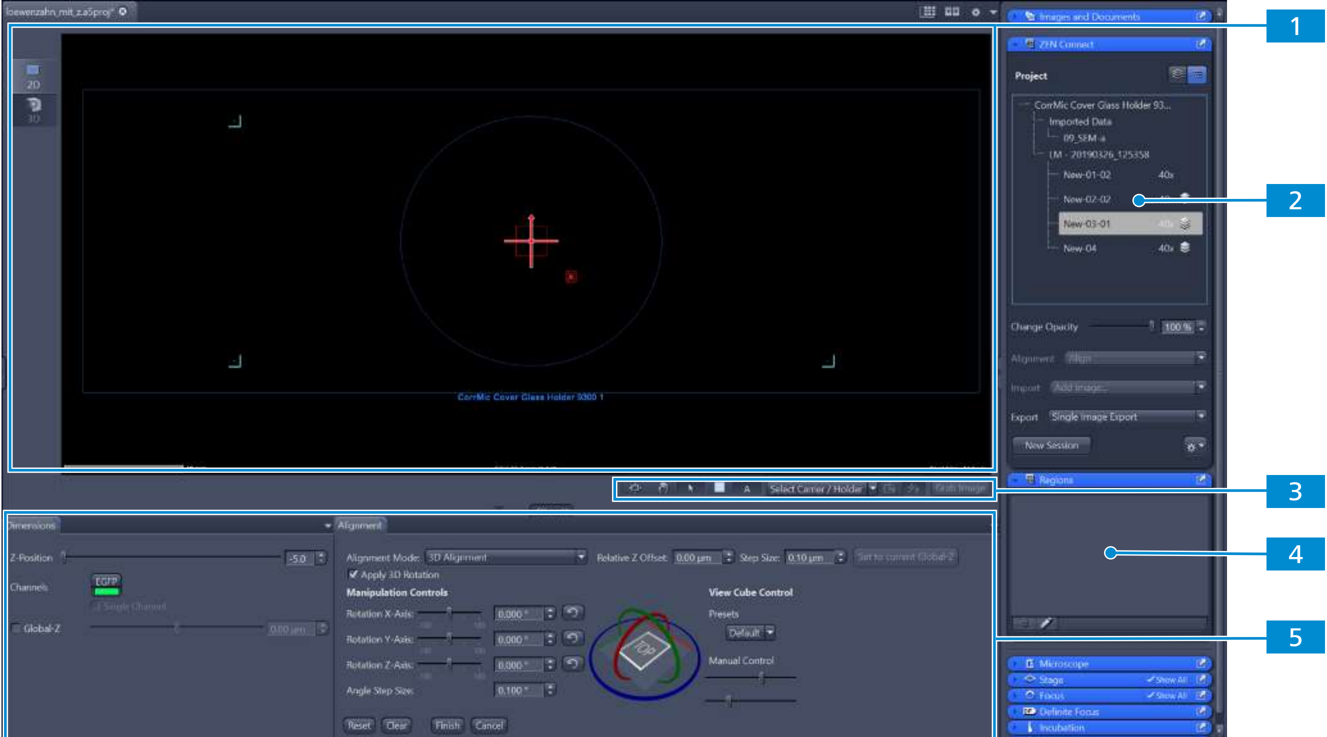
- S&F calibration
- Definition of regions of interest in the correlative workspace
- Retrieval of defined regions of interest

Connect 3D Add-on

This license requires the **Connect** license. Additionally, it includes the following functionalities:

- Control of the displayed z-position in ZEN Connect
- Alignment of images in z-dimension
- View two 3D stacks (requires 3Dxl license)
- Import FIB stacks

12.20.2 Introduction to the User Interface



1 **Image View**
Area where you interact with images. Here, for example, you can select images and align them.
ZEN Connect provides its own 3D viewer. For more information on the user interface of this 3D viewer, see *ZEN Connect 3D View [▸ 564]*. You can use the view tabs on the left side of the Image View to switch between the two viewers.

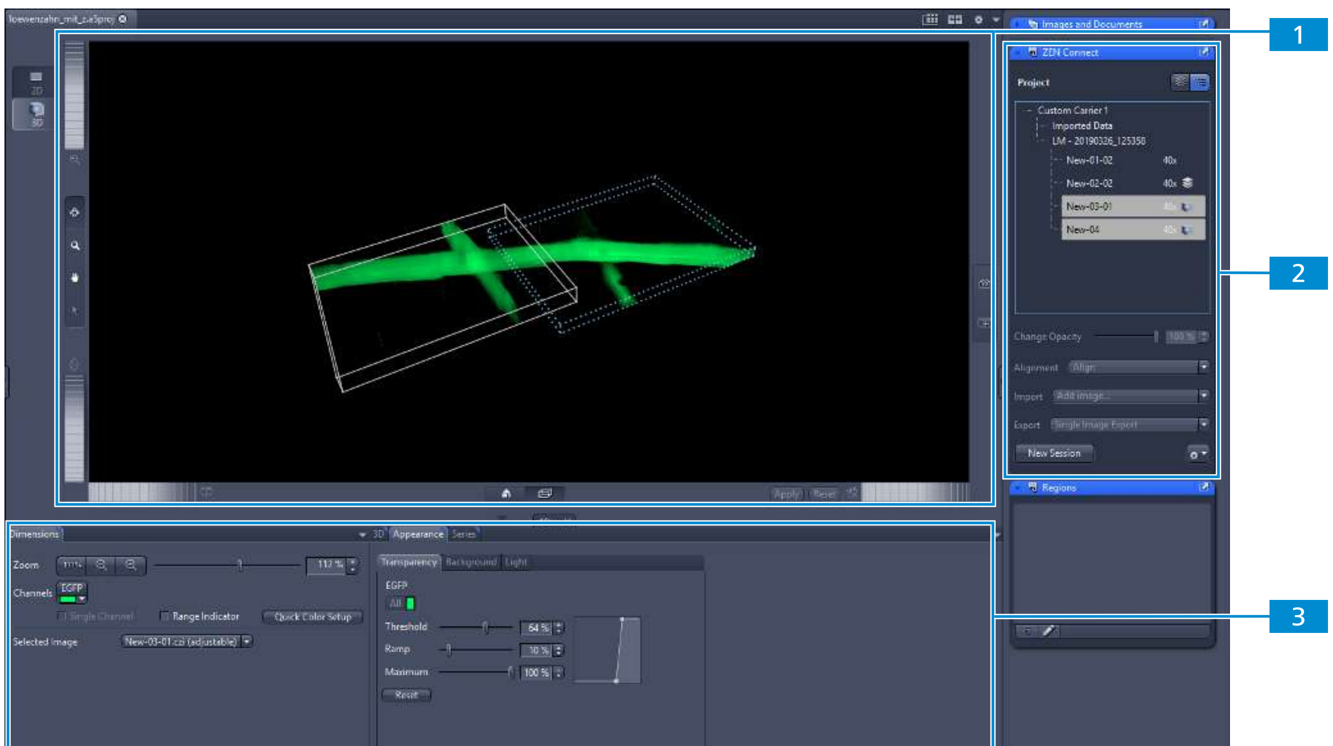
2 **ZEN Connect Tool**
Area where you manage your projects and contained images. For more information, see *ZEN Connect Tool [▸ 601]*.

3 **Button bar below Image View**
Functionalities to pan, zoom, select a region, toggle region captions or view modes, and select a carrier/holder. For more information, see *Button bar below Image View [▸ 597]*.

4 **Regions Tool**
Displays a list of Regions of Interest which are drawn into a ZEN Connect project. For more information, see *Regions Tool [▸ 606]*.

5 **View Options**
Area for general view options of the *Dimensions Tab [▸ 887]* and specific view options on the *Alignment Tab [▸ 595]*. The Alignment tab is only visible when you have entered the mode to align images (see also *Activating the Alignment Process [▸ 576]*).

12.20.3 ZEN Connect 3D View



1 **3D Image View**
Area where you interact with the two 3D volumes and use the options of the three tool bars left, right, and below the Image View. For more information, see *Tool bars [▸ 836]*.

2 **ZEN Connect Tool**
The standard ZEN Connect tool where you manage your projects and contained images. For more information, see *ZEN Connect Tool [▸ 601]*.

3 **View Options**
Area for general view options, like the *Dimensions Tab [▸ 887]*, and the 3D specific view options (*3D Tab [▸ 840]*, *Appearance Tab [▸ 841]*, and *Series Tab [▸ 845]*).
Note: The 3D specific view options do not offer all the functionality compared to the standard 3D view in ZEN.

See also

- Opening images in ZEN Connect 3D view [▸ 565]
- Aligning images in the ZEN Connect 3D view [▸ 584]

12.20.4 Opening the Correlative Workspace

1. Start the software. For more information, see *Starting the Software [▸ 17]*.

The software opens and **ZEN Connect** is available.

Note that before working with **ZEN Connect**, you need to create a **ZEN Connect** project. For more information, see *Creating a ZEN Connect project [▸ 565]*.

12.20.5 Opening images in ZEN Connect 3D view

In the ZEN Connect 3D view you can display two z-stacks as 3D volumes and interact with them. Note that a single image or more than two images cannot be displayed in this 3D view.

Prerequisite ✓ You have opened a ZEN Connect project with at least two z-stacks.

1. In the tree view of the **ZEN Connect** tool, select two z-stacks you want to display in the 3D view.
   Note: The order in which you select the two stacks is important! The stack selected first will be labelled as **fixed** in the 3D view and the second selected stack as **adjustable**. Only the adjustable one can later be aligned in the 3D view and the fixed image serves as a reference.
2. Right click and select **Show in 3D**. Alternatively, click on the context menu button and select **Show in 3D**.

The ZEN Connect 3D view opens and displays the two selected z-stacks as 3D volumes. The two stacks displayed in the 3D view are marked with an icon in the tree view of the **ZEN Connect** tool.

See also

Aligning images in the ZEN Connect 3D view [584]

12.20.6 Non Image Data

In Zen Connect, it is also possible to import non image data into your project, have a visual representation (marker) of it in your image area, and align the position of the data marker with respect to the images in the project. The data is listed under **Non-Image Data** in the tree of the **ZEN Connect** tool and represented by the marker in the image area. Per default, the marker for this non image data is toggled invisible. To toggle data visible and invisible, see also *Moving or hiding images [570]*.

See also

Importing non image data [574]
Aligning non image data [586]

12.20.7 Project and Image Management

12.20.7.1 Creating a ZEN Connect project

Within a **ZEN Connect** project, in the **ZEN Connect** tree view, you manage your data in a project structure tree combined with the viewer. Before acquiring or importing any images, you need to create the **ZEN Connect** project. Only within a **ZEN Connect** project, you can use all **ZEN Connect** functionality.

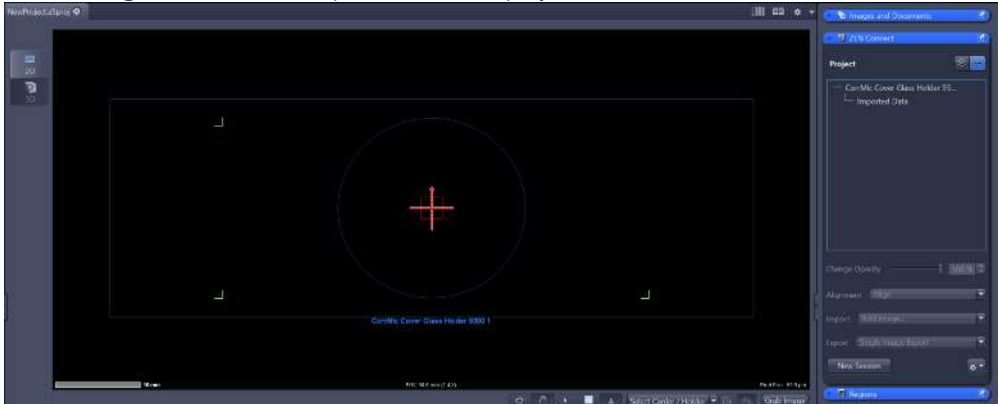
You can open only one **ZEN Connect** project at a time.

Prerequisite ✓ You have set up the sample on the microscope.

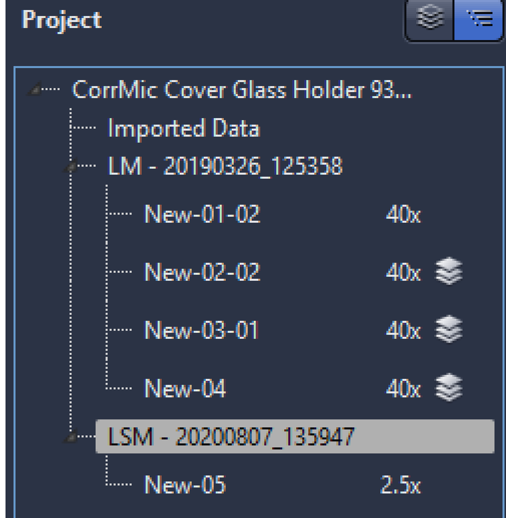
1. In the **ZEN Connect** tool, press the **Create** button.

   → The *New Document Dialog [616]* dialog opens.

2. In the **New ZEN Connect Project Setup** area, select the **Project Path** where you want to store the **ZEN Connect** project file.
3. Select the relevant data to configure the **ZEN Connect** project. If you select the holder/carrier now, you can change it later. Note: If you change the holder/carrier after a S&F calibration, the S&F calibration needs to be redone.
4. Click on **OK**.
   → The ZEN Connect project is created with the project file name **<Projectname>.a5proj**. Note that all new images are saved in the subordinated folder <`Projectname_data`>. You can always check the path on the **Acquisition** tab in the **Auto Save** tool. Alternatively, right- click on the project container in the Center Screen Area and select **Open Containing Folder**.
   → In the **Image View** , the sample holder is displayed.



   → In the **ZEN Connect** tool, the empty **ZEN Connect** project is displayed. Here, the structure of the **ZEN Connect** project will be displayed as soon as you acquire or import images. In the folder on your computer, the **ZEN Connect** project file `<CWS project name>.a5proj` is generated. A `<ZEN Connect project name>.a5lock` is generated to prevent more than one user to work on the project at the same time. It is generated any time you load a **ZEN Connect** project.
   → At the bottom of the **Image View**, a scale bar with size, the width of the field of view (FOV), and scaling is displayed.
   → You have created a **ZEN Connect** project.
5. Acquire an image.
   → In the **Project View**, a new session node is created, and each acquisition is displayed.



   → In the **Image View**, all images are displayed. They are signed with a colored frame (blue: normal image, red: selected image).
6. When you close the project or the software, you are prompted to save the project file.

For information on setting up holders and carriers, see *Selecting and clearing carrier / holder [▸ 589]*.
For information on the **ZEN Connect** Tool, see *ZEN Connect Tool [▸ 601]*.

12.20.7.2 Loading a ZEN Connect project

You can load any of your **ZEN Connect** projects to continue with your work. You can also load existing ZEISS Atlas 5 projects.

Info

Open first the **ZEN Connect** project before performing a S&F calibration.

Prerequisite ✓ You have created a **ZEN Connect** project, or an ZEISS Atlas 5 project is available. ZEISS Atlas 5 projects belong to the ZEISS ATLAS 5 software. **ZEN Connect** supports these formats.

1. Select **File > Open**, navigate to the **ZEN Connect** project and open it.

In the **ZEN Connect Project View**, the current state of the **Connect project** is displayed. In the **Image View**, the sample holders are marked, and previously acquired images are displayed. The current stage position is marked with a cross hair. If you want to acquire additional images to the project, align the new session with the existing data.

See also

Opening or deleting a ZEN Connect project from the data storage [▸ 593]

12.20.7.3 Adding an image to the ZEN Connect project

You can import simple images, such as camera images or more complex images, such as a light microscope image with overlays, into your **ZEN Connect** project.

You can use an imported image as a backdrop to navigate the region. You can correlate imported images with sample holder marks, e.g., fiducials or other images through the alignment process. The imported image is displayed according to its position in the **Layers View** along with any other image in the project.

The metadata of .CZI-images are read natively and are also imported.

Prerequisite ✓ You have loaded a **ZEN Connect** project.

1. In the **ZEN Connect** tool in the on **Project View** , right-click a session and select **Add image**. Alternatively, for the **Import** button, select **Add image**. If you want to add an image from ZEN Data Storage (without downloading it), right click in the **Imported Data** and select **Add image from storage**.
   → You are prompted to select the image you want to add.
2. If you want to import the image as a background image (at the lowest level of the project structure), activate the **Background Image** checkbox.
3. You also have the option to copy the image in a subfolder of the file where the project data is stored before adding it to your project. Activate **Copy files**.
4. Click on **Open**.

The image is imported to your **ZEN Connect** project.

In the **Project View**, it is displayed in the tree, subordinated to **Imported Data**.

If Shuttle & Find-calibration is available for the image and the image is at least in one session in the **ZEN Connect** project, the image is placed in the **Image View** according to the Shuttle & Find stage position. Take care that you select the correct sample holder when importing an S&F-calibrated image. The correct sample holder is the same sample holder that was used during acquisition.

If no Shuttle & Find data is available, the image is displayed centrally in the FOV of the **Image View**.

To add an open image to a project, see *Adding an open image to the ZEN Connect project [▸ 568]*, and to add a dataset, see *Adding datasets when adding images [▸ 569]*.

Additionally, you have the following options to import data in your **ZEN Connect** project:

- *Importing Data [▸ 572]*
- *Importing Third-party images [▸ 572]*
- *Importing a SmartFIB stack into ZEN Connect [▸ 573]*
- *Saving an image to the data storage [▸ 592]*
- *Importing non image data [▸ 574]*

Note: If you import an Airyscan image, **Zen Connect** displays only the raw data and not the calculated Airyscan. Such images should be processed before you add them to **Zen Connect**. If you want to add an unprocessed Airyscan image, a warning will appear asking if you want to continue.

Note for z-stacks: When you use the **Global Z** slider and are beyond the range of a certain image, only a frame for this image is displayed to show where the image is positioned.

12.20.7.4 Adding an open image to the ZEN Connect project

Prerequisite
✓ You have loaded a **ZEN Connect** project.
✓ You have an open image (which is not part of the project).

1. Click on **File > Add to ZEN Connect Project**.
   Alternatively, you can use the button in the tool bar which is available if you customize your application by clicking on **Tools > Customize Application... > File >** and double click **Add to ZEN Connect Project**. This button is automatically available in the tool bar for all users of the **EM Processing Toolbox** module.

The image is added to your **ZEN Connect** project.

Additionally, you have the following options to import data in your **ZEN Connect** project:

- *Importing Data [▸ 572]*
- *Importing Third-party images [▸ 572]*
- *Importing a SmartFIB stack into ZEN Connect [▸ 573]*
- *Saving an image to the data storage [▸ 592]*

Note: If you import an Airyscan image, **Zen Connect** displays only the raw data and not the calculated Airyscan. Such images should be processed before you add them to **Zen Connect**. If you want to add an unprocessed Airyscan image, a warning will appear asking if you want to continue.

Note for z-stacks: When you use the **Global Z** slider and are beyond the range of a certain image, only a frame for this image is displayed to show where the image is positioned.

See also

Adding an image to the ZEN Connect project [▸ 567]

12.20.7.5 Adding datasets when adding images

Optionally, you can add datasets to organize your imported data.

Prerequisite ✓ A **ZEN Connect** project is loaded.

1. In the **Project View** tree, select right-click **Imported Data** and select **Add dataset**. Alternatively, in the **ZEN Connect** tool, select **Add dataset** for the **Import** button.
2. Navigate to the image you want to add. Select it, and click **Open**.

The image is included in your **ZEN Connect** project, located below **New dataset**.

12.20.7.6 Zooming images

You can zoom images in and out of a field of view (FOV).

Zooming to

Prerequisite ✓ A **ZEN Connect** project is loaded.

1. In the **Project tree View**, right-click an image and select **Zoom to**. Alternatively, click on the **Context menu** button and select **Zoom to**.

The image is centered in the view space of the **Image View**.

Zooming to 100%

Prerequisite ✓ A **ZEN Connect** project is loaded.

1. In the **Project tree View**, right-click an image and select **Zoom to 100%**. Alternatively, click on the **Context menu** button and select **Zoom to 100%.**

The image is zoomed in the view space of the **Image View** to 100%.

12.20.7.7 Removing images from the ZEN Connect project

You can remove images from your **ZEN Connect** project. Images you acquired and added to the project, you can also delete permanently from the disk.

Prerequisite ✓ You have loaded a **ZEN Connect** project.

1. In the **Project** view or in the **Layer** view, select the images to remove.
2. Right-click the image, and select **Remove data**. Alternatively, click on the **Context menu** button and select **Remove data**. If you want to remove an acquired image, you are asked if you also want to delete it from the disk.

The images are removed from the **ZEN Connect** project. If you remove all images from the folder, the folder is removed as well. Removing data cannot be undone.

See also

ZEN Connect Tool [▸ 601]

12.20.7.8 Renaming images in a ZEN Connect project

Prerequisite ✓ You have loaded a **ZEN Connect** project.

1. In the **Project** view or in the **Layer** view, select an image to rename.
2. Right-click, select **Rename data** and rename the image, or press the *F2* key. Alternatively, you can double click the image name to rename it, or click on the **Context menu** button , and select **Rename data**.

You have renamed the image. It is updated either in the **Layer** or the **Project** view respectively.

The image name is not changed on the disk.

12.20.7.9 Opening images in ZEN

Prerequisite ✓ You have loaded a **ZEN Connect** project.

1. In the **Project View** or in the **Layers View**, select one or more images you want to open, and right-click **Open image(s) in ZEN**. Alternatively, click on the **Context menu** button and select **Open image(s) in ZEN**. Additionally, you can also simply double click on an image in the **Project View** or in the **Layers View**.

The image is opened in ZEN and displayed on a separate tab.

NOTICE

ZEN Data Storage

If you have opened a project from ZEN Data Storage, you can also open an individual image this way. The image is then downloaded and each change is not updated in the viewer and the project until you upload the image again. For information on uploading an image to the data storage, see *Saving an image to the data storage* [▸ 592].

12.20.7.10 Showing an image in the Explorer

You can locate an image on your computer.

Prerequisite ✓ You have loaded a **ZEN Connect** project.

1. In the **Project View** or in the **Layers View**, right-click an image and select **Show in Explorer**. Alternatively, click on the **Context menu** button and select **Show in Explorer**.

The explorer opens and the folder with the selected image is displayed.

12.20.7.11 Moving or hiding images

In the **Layers View**, you can move images over and under other images, or hide them completely. In the **Project View**, you can hide images.

Prerequisite ✓ You have loaded a **ZEN Connect** project with at least two images.

1. To change the image order, in the **Layers View**, move the image by dragging it up or down.
   → In the **Image View**, the changed order is immediately visible.

2. To hide the image from the **ZEN Connect** project, in the **Layers View**, activate or deactivate the image by clicking the **Eye** icon on the right of the image name. Alternatively, in the **Project View**, right-click **Show/Hide** or click on the **Context menu** button and select **Show/Hide**.

The results of your changes are displayed immediately in the **Image View**.

See also

ZEN Connect Tool [▸ 601]

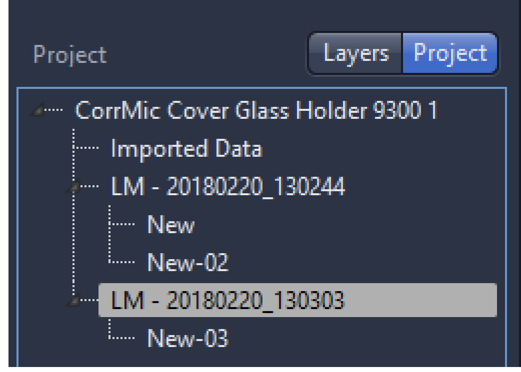
12.20.7.12 Starting a new session

To organize your work, you can start a new session within your **ZEN Connect** project any time.

Prerequisite ✓ You have loaded a **ZEN Connect** project.

1. In the **ZEN Connect** tool, click on the **Context menu** button and select **New session**.
   Alternatively, click on the **New Session** button.

A new session is activated. As soon as you acquire a new image, a new session node is created, and the new image will be subordinated.



See also

ZEN Connect Tool [▸ 601]

12.20.7.13 Toggling view modes

In ZEN Connect you can switch between two different view modes for your projects. The default is the carrier/ holder view mode, where the coordinate system of the correlative workspace is aligned with the screen and images of the current system/ session might be rotated. The second is the stage centric view mode, where the coordinate system of the current session is aligned with the screen and the carrier/ sample holder as well as other sessions might be rotated.

Prerequisite ✓ You have opened a ZEN Connect project.

1. In the button bar below the **Image View** of the correlative workspace, click on the button for the carrier/ holder or the stage centric view mode.

The view is changed according to the selected view mode.

12.20.7.14 Closing a ZEN Connect project

You have the following options to close a **ZEN Connect** project:

1. Click on **File > Close**
2. In the **Image View**, click on the cross next to the **ZEN Connect** project name.

12.20.8 Import and Export

12.20.8.1 Importing Data

You can import simple images, such as camera images or more complex images, such as a light microscope image with overlays, to your **ZEN Connect** project. For more information, see *Adding an image to the ZEN Connect project [567]*.

Alternatively, you have the option to import BioFormats into your **ZEN Connect** project. For more information, see *Importing Third-party images [572]*.

Note: If you import an Airyscan image, **Zen Connect** displays only the raw data and not the calculated Airyscan. Such images should be processed before you add them to **Zen Connect**. If you want to add an unprocessed Airyscan image, a warning will appear asking if you want to continue.

See also

ZEN Connect Tool [601]

12.20.8.2 Importing Third-party images

ZEN uses BioFormats as an integrated library for reading and writing life sciences image file formats. It is capable of parsing both pixels and metadata for a large number of formats. It achieves this by converting proprietary microscopy data into an open standard called the OME data model. With BioFormats, you can read proprietary formats and convert them into an intermediate format, e.g., CZI or OME-TIFF. For example, it is possible to load simple images you can import simple images, such as camera images or more complex images, such as a light microscope image with overlays, to your **ZEN Connect** project.

Prerequisite ✓ You have licensed the Third Party Import.

1. Select **File > Export/Import > BioFormats Import**.
2. Navigate to the image with the proprietary image format you want to import, select it, and click **Open**.

The image is converted and added to **ZEN** (not to the **ZEN Connect** project itself).

Alternatively, you have the following options to import data in your **ZEN Connect** project:

- *Adding an image to the ZEN Connect project [567]*.
- *Importing Data [572]*

For more information on BioFormats, see http://www.openmicroscopy.org/bio-formats/

12.20.8.3 Importing a SmartFIB stack into ZEN Connect

With ZEN Connect you can import SmartFIB stacks of crossbeam microscopes and align them with data from light microscopes. The orientation of these stacks slightly differs from standard z-stack acquisition, as the acquired images are tilted by a certain angle compared to a usual z-stack. The import calculates this tilt from the metadata of the image. If the import finds no metadata concerning the tilt angle and the user does not enter a value, it uses a default of 90 degrees. Alternatively, you can enter the angle of your sample during import, if you know it, and the import then calculates the tilt angle based on this sample angle.
During import, the XY offset metadata of the individual slices is ignored by default and only the offset of the first tiff file is considered. This default avoids the creation of a slanted z-stack in case some slices contain incorrect metadata. Activate the checkbox **consider individual slice offsets** on the import dialog to change the behavior.

Prerequisite ✓ You have opened/created a ZEN Connect project.

1. In the **Project** view, select the session where you want to import the FIB stack.
2. Right click on the session and select **Import SmartFIB z-stack**.
   Alternatively, select **Import SmartFIB z-stack** for the **Import** button.
   → A file browser opens.
3. Select the images you want to import as FIB stack.
   Note: Select only images which belong to one dataset!
   Note: To make sure the stack is composed/ ordered correctly, watch out how the images are sorted in the explorer and in which order you choose them.
4. You can enter a name for the FIB stack. This step is optional.
5. If you import images without scaling information, you can manually enter them as **optional Z-stack XY scaling**.
   Note: ZEN currently cannot determine automatically if scaling information are present!
6. You can set the slice distance. This step is optional and should only be done if you have reason to believe the information calculated with the metadata is incorrect. Leave the input field empty and the slice distance is automatically calculated with information saved in the metadata of the images.
   Note: When you set the slice distance manually, the information in the metadata is ignored.
7. If you know the angle of your sample, enter it under **optional sample angle**. Otherwise the tilt for the image is calculated with the metadata or set to the default of 90 degree (if no information is available in the metadata).
8. If you want to consider the XY offset metadata of the individual slices for the import, activate the checkbox **consider individual slice offsets**. Note that this can lead to a slanted z-stack if the metadata contain incorrect offset information!
9. Click on **Open**.

The FIB stack is now imported into the selected session.
Note: When importing larger image files it may take a while until the entire stack is visible in the viewer. This also applies when you open a project that contains such larger stacks.

Note: To improve the alignment of the z-stack, the TrakEM2 format is supported. Before importing the SmartFIB z-stack, use a specific fiji script (for information, see https://imagej.net/Register_Virtual_Stack_Slices#API_documentation) that creates xml-files with TrakEM2 format within the passed input folder. Those xml-files are then considered during the import and replace the stage-position information with the computed pixel delta x/y shifts. Only the stage-position of the first image is used for absolute positioning, all other images of the stack are positioned according to this first slice and the computed shifts.

12.20.8.4 Importing non image data

Prerequisite ✓ You have opened/created a ZEN Connect project.

1. In the **ZEN Connect** tool, select **Import non-image data** for the **Import** button.
   → A file browser opens.
2. In the file browser, select your file. If the file should be copied into the data folder of the project, make sure the checkbox **Copy Files** is activated (it is activated by default). If the file should only be linked into the project, deactivate the checkbox.
3. Click on **Open**.

You have successfully imported the non-image data into your ZEN Connect project. The file is listed under **Non-Image Data** in the tree of the **ZEN Connect** tool.

See also

Non Image Data [565]

12.20.8.5 Exporting single image data

You can export data of **ZEN Connect** projects as a single image for distribution to collaborators, or for the use in publications. The content can be a single image, tiles, a collection of images, or a view of the entire **ZEN Connect** project. You can drag or resize the region to control the area that you want to export, or activate if image names and frames are shown on the exported image or not. You can pan and zoom using the mouse in the **Image View** to get fine control of the export area.

Prerequisite ✓ You have loaded a **ZEN Connect** project.

✓ In the loaded **ZEN Connect** project, you have activated and deactivated the respective areas of interest.

1. In the **ZEN Connect** tool, open the **Project View** or the **Layers View**. Right-click an image and select **Single Image Export**.
   Alternatively, select an image and select **Single Image Export** for the **Export** button.

   → A wizard opens.
2. Make your settings and click **Export Data**.
3. Navigate to the folder where you want to store the exported image. The default file name is the **ZEN Connect** project name. Click **Save**.

You have exported one image in a standard image format. The exported image is based on the export area you set up in the **Image View**.

See also

Configure single image export wizard [597]

ZEN Connect Tool [601]

12.20.8.6 Exporting a Zen Connect project as a video

You can export data of **ZEN Connect** projects as a video.

Prerequisite ✓ You have loaded a **ZEN Connect** project.

✓ In the loaded **ZEN Connect** project, you have activated and deactivated the respective areas of interest.

1. In the **ZEN Connect** tool, open the **Project View** or the **Layers View**. Right-click an image and select **Video Export**.
   Alternatively, select an image and select **Video Export** for the **Export** button.

   → The wizard for video export opens.
2. Choose your key frames by positioning the export area in the **Image View** and add them to the list of key frames by clicking **Add current view as key frame**.
3. Make your settings, and click **Export Data**.
4. Navigate to the folder where you want to store the exported video. The default file name is the **ZEN Connect** project name. Click on **Save**.

You have exported a video in a standard video format.

See also

ZEN Connect Tool [601]
Video Export wizard [599]

12.20.8.7 Exporting data for SerialEM

ZEN offers the functionality to export image data as a MRC file which makes it compatible to the software application SerialEM and available for TEM users. This export is available for z-stacks and multi channel images with all the common pixel types (8/16 Bit, 32 Bit Float, RGB 24/32/48). Tiles, time series, multi-scene images, and images with unprocessed data or special dimensions are not supported. For images with more dimensions, you can use the image processing function *Create Image Subset [112]* to extract a single image or stack and then export it with this function. The export creates a MRC file as well as a NAV file, which contains regions of interests (e.g. points, rectangles, polygons,...). The NAV file can be loaded in the SerialEM software and it then loads the MRC file, so that the image with the respective regions is shown.

Prerequisite ✓ You have opened an image in ZEN.

1. Click on **File > Export/Import > MRC Export**.
   → A file explorer opens.
2. In the file explorer, select a folder to save the file.
3. Name the file and click on **Save**.

You have exported the image data as a MRC (and NAV) file.

12.20.8.8 Exporting a ZEN Connect project from the data storage

In ZEN, you can export a ZEN Connect project from the ZEN Data Storage to use it locally (e.g. on a machine with no access to the database).

Prerequisite ✓ You have opened your project from the database in ZEN. For more information, see *Opening or deleting a ZEN Connect project from the data storage [593]*.

1. In the **ZEN Connect** tool, select **Export Project...** for the **Export** button.
2. In the file browser, select the location where you want to export your project.
3. Click on **OK**.
   → Your project is exported to the selected location. The state of the export is displayed in the progress bar. For each exported project a subfolder is created in the selected export location.

You have successfully exported your project from the data storage.

12.20.9 Handling of images and data

12.20.9.1 Alignment

The module Shuttle & Find allows the correlation of two images. For more information on Shuttle & Find, see Module Shuttle & Find.

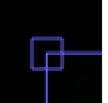
Additionally to that functionality, in a **ZEN Connect** project, you can manually align images in your workspace to correct their position or size with respect to the samples. Within a **ZEN Connect** project, you can calibrate your system using a sample holder with fiducial markers by moving between the markers and confirming their positions. To do so, you activate the alignment process and start aligning image data.

12.20.9.1.1 Activating the Alignment Process

The alignment process lets you align your current session with fiducial marks or previous images. You can align image data manually.

You should create a new session any time the alignment of the sample in the microscope has been disturbed.

Prerequisite ✓ A ZEN Connect project is loaded.

1. In the **Layer View**, or in the **Project View**, select the image you want to align. Alternatively, you can select a region to select a couple of images.
   → The image is marked with a square in each frame corner.
   
   → As long as the alignment process is not activated, this is indicated with a little lock next to the cursor.
2. Right-click the selected image and select **Align Data**.
   Alternatively, right-click the image(s) in the **ZEN Connect** tool and select **Align Data**.You can also select **Align** for the **Alignment** button and click it.

You have activated the alignment process for one or more images. The *Alignment Tab [▸ 595]* below the **Image View** is displayed.

You can start aligning image data. If you start an alignment on a session node, the set alignment is used for all current and future images of the session. You can use this if you change your sample between different systems and want to align their coordinate systems to each other.

See also

ZEN Connect Tool [▸ 601]

12.20.9.1.2 Aligning image data

In the alignment process, you have various options to align image data. Note that you can change the alignment mode during the alignment process. The alignment edits you have made are preserved, but you have to restart the pinning process if you have inserted any pins before changing the mode.

Note: The alignment process can be executed multiple times. Each time you run the alignment process, the end result of the last alignment is used as the starting point for the new alignment. If the initial image was far out of alignment at the start, it is easiest to do the alignment process

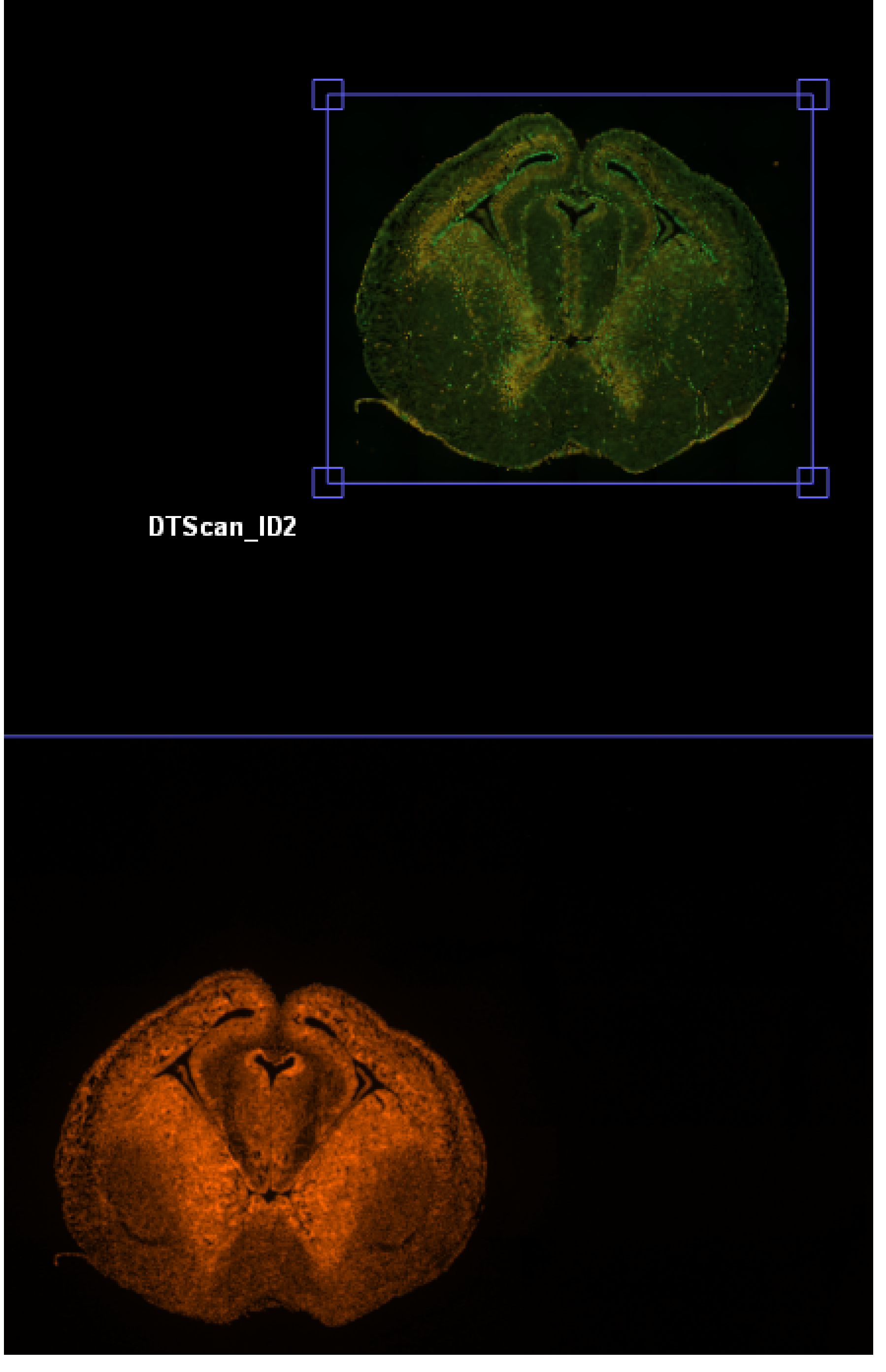
once roughly, and then do the alignment process a second time with more precision. The second alignment will use the first alignment as a starting point, and will allow you to establish a more precise alignment quickly.

Prerequisite ✓ You have loaded a ZEN Connect project and activated the alignment process.

1. In the **Alignment** tab, select one of the following alignment modes and the region you want to align.

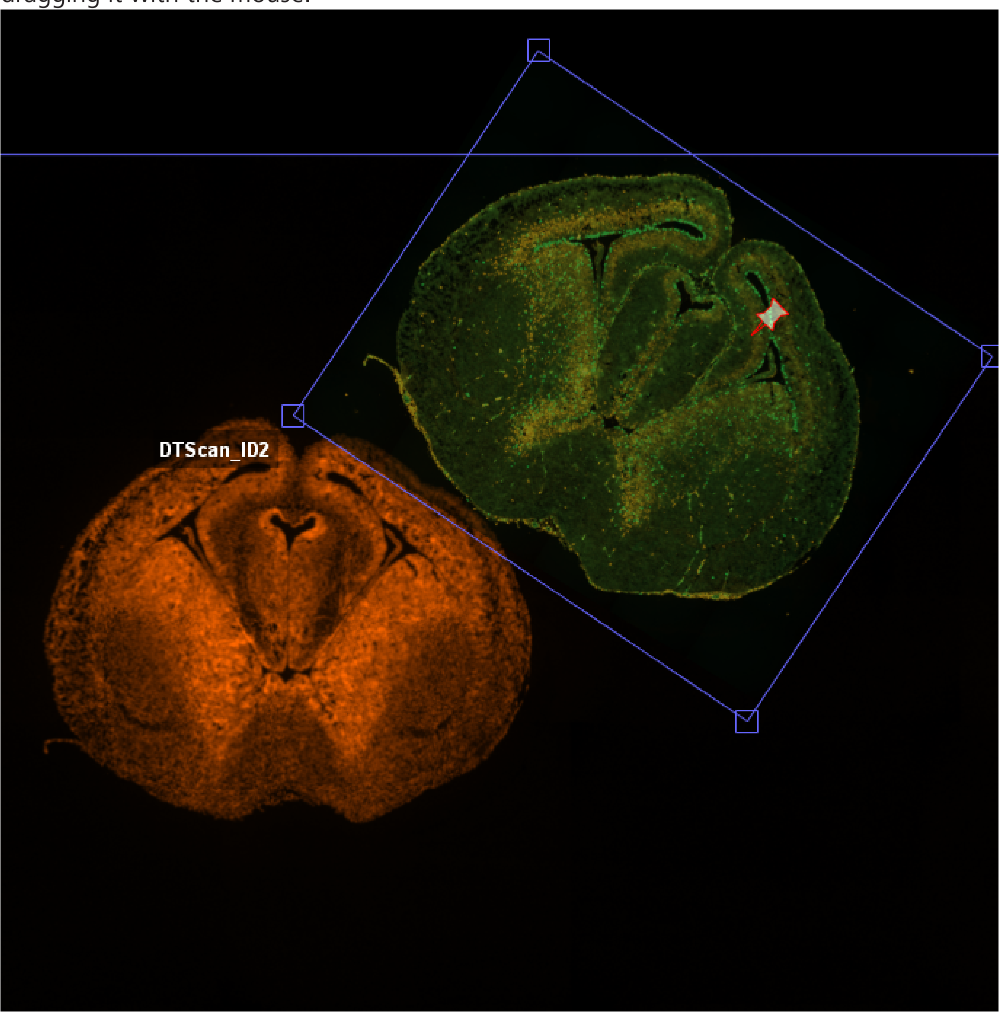
Translate Only

1. Click and drag with the mouse to translate the image you are aligning with respect to everything else.
   → You can zoom in and out with the mouse wheel, or press and hold the *CTRL* key to pan while you are in the process of aligning the image.



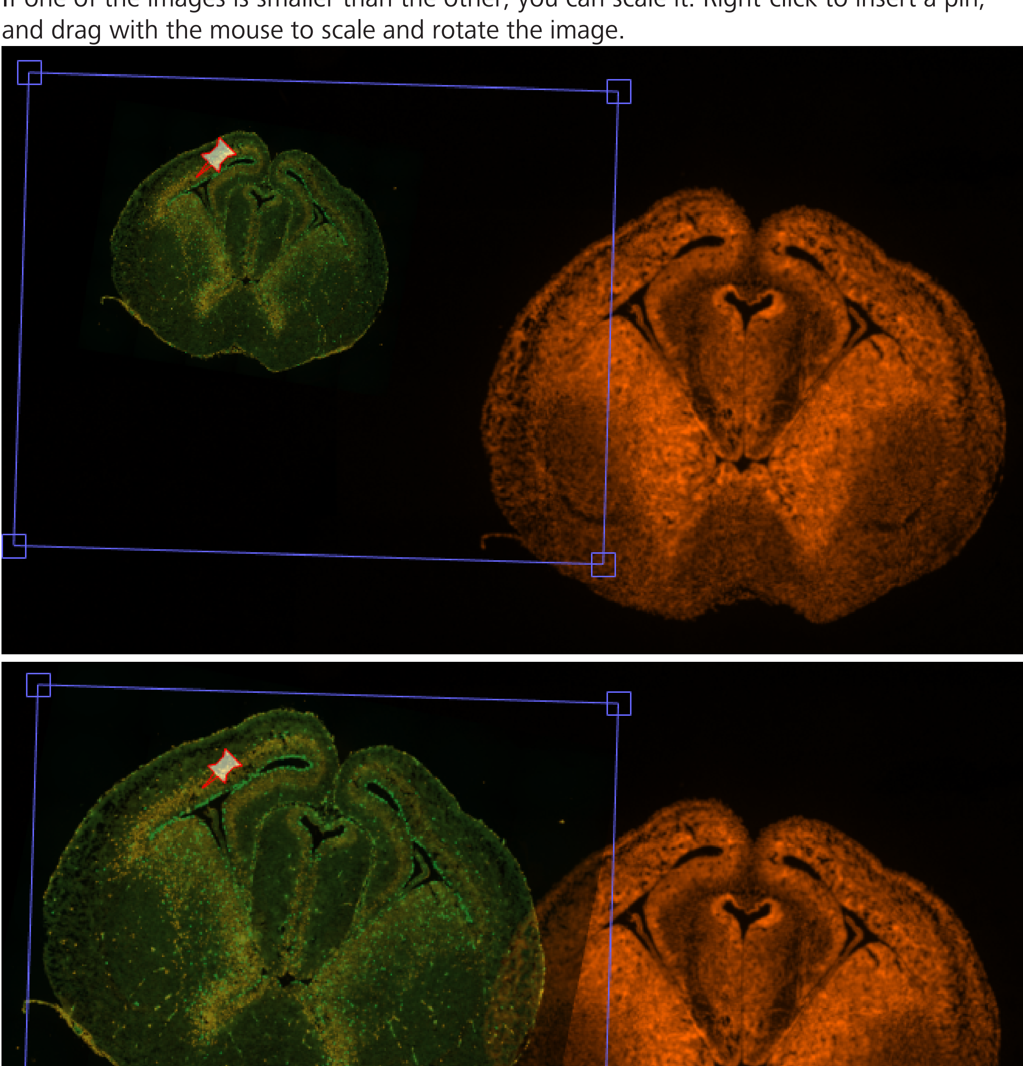
Translate and Rotate Only

1. Right-click at the location you have lined up to insert the first pin, a red and grey pin icon. The pin locks the image to the reference at this location. Press the *DEL* key to remove the last pin you inserted.
   → After you insert the first pin, your input will rotate the item around the first pin, when dragging it with the mouse.



Translate, Rotate and Scale Only

1. If one of the images is smaller than the other, you can scale it. Right-click to insert a pin, and drag with the mouse to scale and rotate the image.



Translate, Rotate, Scale and Shear

1. Right-click to insert a second pin, and drag with the mouse to shear the image.
   → After you insert the second pin, your input will also stretch and shear the item.

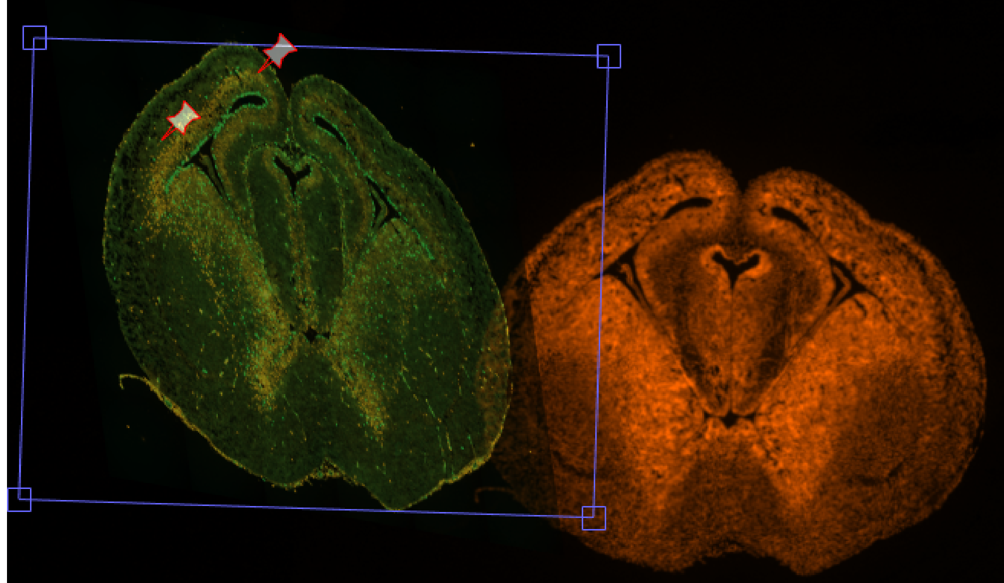
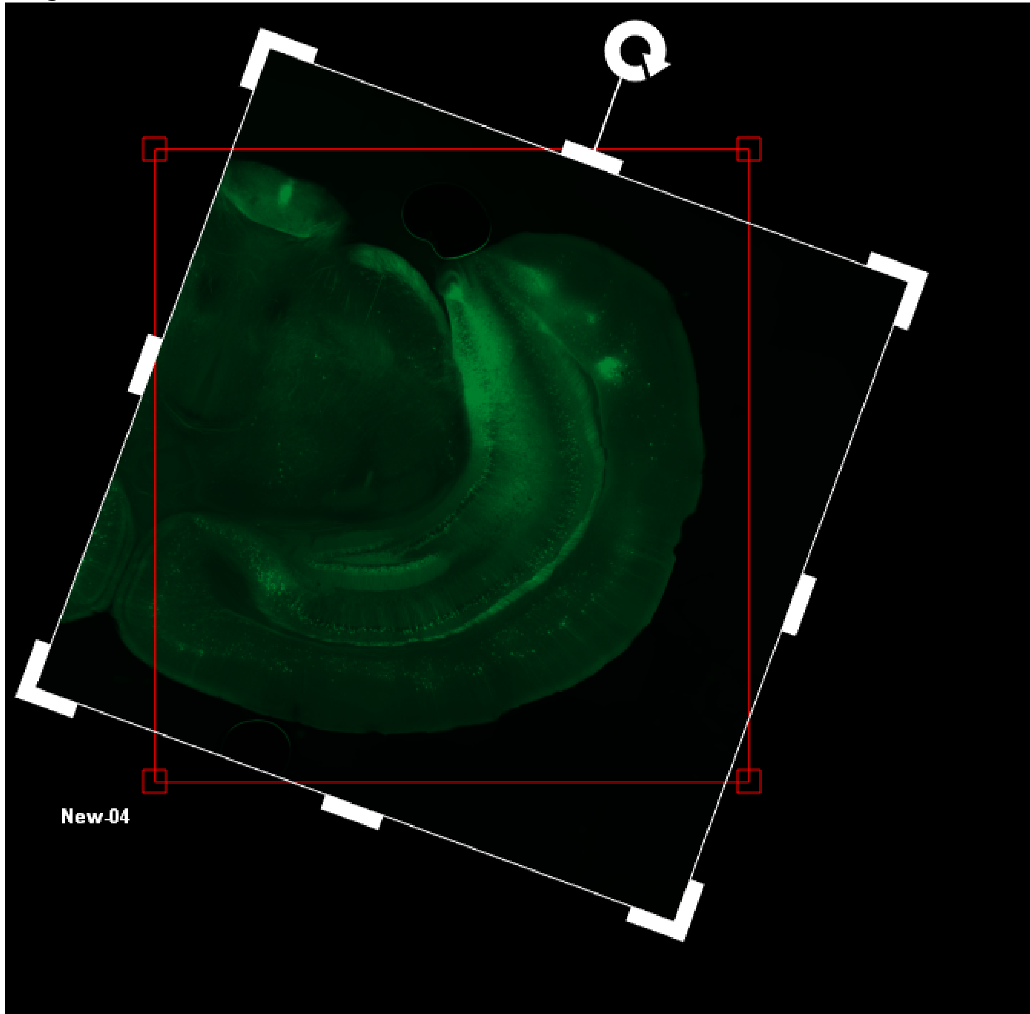


Image data from microscopes should not need to be stretched or sheared to perform alignment. If you need to provide much input after inserting the second pin, this might be an indication of other problems, such as equipment calibration issues.

Alignment Handles

1. If you select **Alignment Handles**, you can use handles to rotate, translate, and scale the image.



Flipping the image horizontally and vertically

You can flip your image, to mirror it.

- To flip the image horizontally, click on the **Flip Horizontally** button .
- To flip the image vertically, click on the **Flip Vertically** button .
- To flip the image stack in z direction, click on the **Flip in Z** button .

Reset alignment

1. Click on the **Reset** button to reset the alignment you performed.
   → The alignment is reverted as it was when you started aligning. The alignment mode ist still activated.

Cancel alignment

1. Click on the **Cancel** button to reset the alignment you performed.
   → The current alignment is cancelled and reverted to the alignment in place before you started the alignment mode. The alignment mode is not activated any longer.

Finish alignment

1. Click on the **Finish** button to finish the alignment mode and to save the alignment information.

Clear alignment

1. Click on the **Clear** button.
   → The session is restored to it's un-aligned state.

See also

- Aligning images in z direction [▸ 582]
- Rotating a z-stack [▸ 584]
- Alignment Tab [▸ 595]

12.20.9.1.3 Aligning images in z direction

In ZEN Connect you can not only align your images/ sessions in x and y direction, but also in z.

Prerequisite ✓ You have opened a ZEN Connect project containing images/ z-stacks with z information.

1. Select the image or session you want to shift in z direction.
2. In the **ZEN Connect** tool, select **Align** for the **Alignment** button and click it. Alternatively, right click on the image and select **Align Data**.
   → The *Alignment Tab [▸ 595]* is displayed below the Image View.
3. For **Alignment Mode** select **3D Alignment** in the dropdown list.
4. For **Relative Z Offset** set the value for your shift in z direction.
5. Click on **Finish**.

You have now aligned your data in z direction. For an illustration of the alignment see *Example for z alignment [▸ 583]*.
Note that for the z alignment the view of the aligned stack remains the same, whereas the view of the other stacks changes.

Setting an image to the current Global Z

You can also set the center of a z-stack to the currently selected z-position of the **Global-Z** slider.

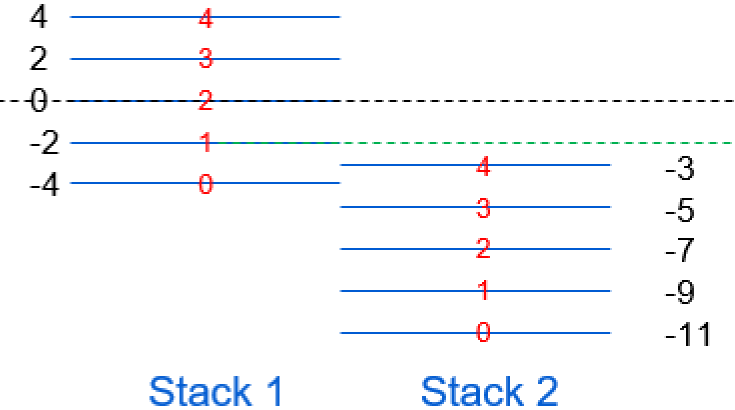
Prerequisite ✓ You have opened a ZEN Connect project containing images/ z-stacks with z information.

1. Activate the **Global-Z** slider and move to the z-position where you want your image to be placed.
2. Select the image you want to shift in z direction.
3. In the **ZEN Connect** tool, select **Align** for the **Alignment** button and click it. Alternatively, right click on the image and select **Align Data**.
   → The *Alignment Tab [▸ 595]* is displayed below the Image View.
4. For **Alignment Mode** select **3D Alignment** in the dropdown list.
5. On the **Alignment** tab, click on the **Set to current Global-Z** button.
6. Click on **Finish**.

You have now set the center of your z-stack to the currently selected **Global-Z**.

12.20.9.1.3.1 Example for z alignment

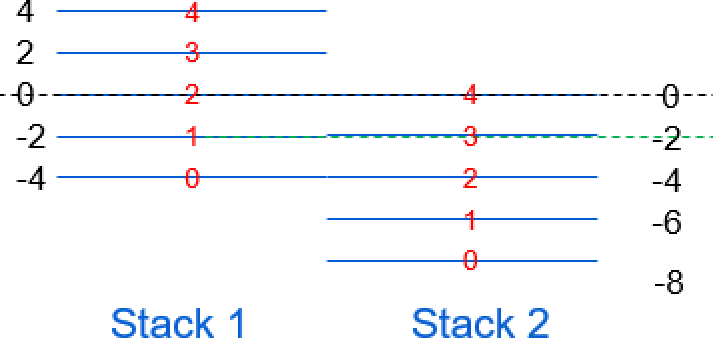
This chapter serves as an illustration of how and what is happening for z alignment in ZEN Connect. Consider the following situation:



This image illustrates two z-stacks with five planes and different z coordinates (with µm as unit). The green line simulates the position of the **Global-Z** slider. Here, the Correlative Workspace would show you plane 1 of the first stack and an empty frame where the second stack would be because the **Global-Z** is beyond the range of the second stack.

Aligning Stack 2 in z direction

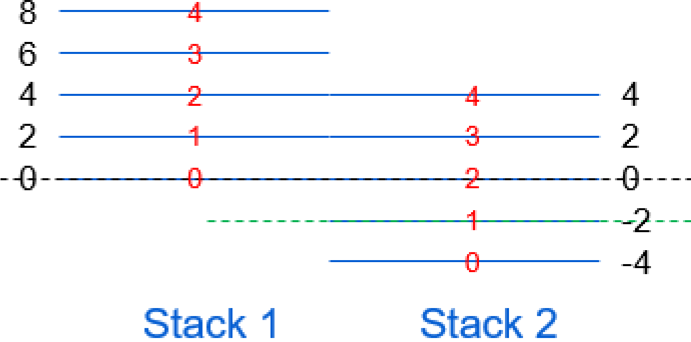
Now the second stack is aligned according to the workflow described in *Aligning images in z direction [▸ 582]* with a **Relative Z Offset** of 3µm. The result then is the following:



All z planes of Stack 2 are shifted by 3µm and now the z-plane 1 of Stack 1 and z-plane 3 of Stack 2 would be visible in the Correlative Workspace.

Aligning the whole session in z direction

Now the whole session with both images is shifted with a **Relative Z Offset** of 4µm.



Now the first stack is out of range of the **Global-Z** slider, so the Correlative Workspace would show you plane 1 of Stack 2 and an empty frame where Stack 1 is located.

See also

Using the Global-Z slider [▸ 591]

12.20.9.1.4 Rotating a z-stack

In the alignment mode you can also perform a three dimensional rotation of a z-stack.

Prerequisite ✓ You have opened a ZEN Connect project.

1. Select the z-stack you want to rotate.
2. In the **ZEN Connect** tool, click on **Align**. Alternatively, right-click the image and select **Align Data**.
   → The *Alignment Tab [▸ 595]* below the Image View is displayed.
3. For **Alignment Mode**, select **3D Alignment**.
4. Activate the checkbox **Apply 3D Rotation**.
   → The **Manipulation Controls** for rotation are activated.
5. Use the respective sliders or input fields to rotate the z-stack around the x, y, or z axis. Alternatively, you can use the **View Cube Control** to rotate the stack interactively.
6. Click on **Finish**.

You have now rotated the selected z-stack around the respective axis.

12.20.9.1.5 Aligning images in the ZEN Connect 3D view

With the ZEN Connect 3D view you can display and align two z-stacks as 3D volumes. You can rotate and translate the volume in this 3D view.
Note: You can only align one of the volumes, which is classified as **adjustable**. The other volume, classified as **fixed**, cannot be aligned. Which volume is the fixed and which the adjustable one depends on the selection in the tree view when you open the 3D view (see also *Opening images in ZEN Connect 3D view [▸ 565]*) and can be seen in the **Dimensions** tab option **Selected Image**.

Translating the 3D volume in x and y direction

Prerequisite ✓ Two z-stacks are opened in the ZEN Connect 3D view.

1. On the **Dimensions** tab, for **Selected Image** choose the volume which is considered as the **adjustable** image.
2. On the left tool bar, click on the arrow button .
3. In the image view, continuously press the middle mouse button and move the mouse to shift the volume in x and y direction.
4. When the volume is in the desired position, stop pressing the mouse button.
5. Click on **Apply** in the tool bar below the image view to apply the changes to the project.

You have now shifted the 3D volume in x and y direction and applied the changes to the project.

Translating the 3D volume in z direction

Prerequisite ✓ Two z-stacks are opened in the ZEN Connect 3D view.

1. On the **Dimensions** tab, for **Selected Image** choose the volume which is considered as the **adjustable** image.
2. On the left tool bar, click on the arrow button .
3. In the image view, continuously press the right mouse button and move the mouse to shift the volume in z direction.
4. When the volume is in the desired position, stop pressing the mouse button.
5. Click on **Apply** in the tool bar below the image view to apply the changes to the project.

You have now shifted the 3D volume in z direction and applied the changes to the project.

Rotating the 3D volume

Prerequisite ✓ Two z-stacks are opened in the ZEN Connect 3D view.

1. On the **Dimensions** tab, for **Selected Image** choose the volume which is considered as the **adjustable** image.
2. On the left tool bar, click on the arrow button .
3. In the image view, continuously press the left mouse button and move the mouse to rotate the volume.
4. When the volume is in the desired position, stop pressing the mouse button.
5. Click on **Apply** in the tool bar below the image view to apply the changes to the project.

You have now rotated the 3D volume and applied the changes to the project.

12.20.9.1.6 Aligning non image data

Prerequisite
- ✓ You have opened a ZEN Connect project with non image data.
- ✓ Your non image data is toggled visible, see also *Moving or hiding images [▸ 570]*.

1. In the **ZEN Connect** tool or in the image view, right-click the non image data and select **Align Point Position**. Alternatively, in the **ZEN Connect** tool, select the non image data and select **Align** for the **Alignment** button and click it.
   → You enter the alignment mode.
2. In the image area, click at the position where you want to place the none image data.
3. Click on **Finish**.

You have aligned your non image data.

See also

Non Image Data [▸ 565]

12.20.9.1.7 Aligning images in the Manual Alignment Wizard

Prerequisite
- ✓ You have opened a ZEN Connect project.

1. In the correlative workspace or in the **ZEN Connect** tool, select the image(s) you want to align.
2. In the **ZEN Connect** tool, for the **Alignment** button, select **Manual Alignment Wizard** and click on it.
   → The *ZEN Connect Alignment Wizard [▸ 608]* opens.
3. To translate the selected image(s), set the distance in x and y direction with the **Translate** parameter section on the left.
   → The translation is immediately displayed in the Image View.
4. To scale the image, set the scaling factor in x and y in the **Scale** section.
   → The scaling is adjusted according to the input.
5. To rotate the image, first select the **Rotation Center** and then enter an **Angle**. The current rotation center is represented in the image by a pin.
   → The selected image(s) is/are rotated.
6. To flip/mirror the image horizontally, click on .
7. To flip/mirror the image vertically, click on .
8. If you want to shear your image, activate **Enable**.
   → Additional shearing pins are displayed in the image. The two pins that form the "baseline" for shearing a connected by a line.
9. Use the two pins to position the baseline and then move the pinpoint to shear the image.
10. Click on **Finish** to save the alignment and close the wizard.

You have aligned the selected image(s) in the ZEN Connect project.

NOTICE

Alignment Handles

You can also use the alignment handles of the selected image(s) to translate, scale, and rotate the image.

NOTICE

View Options

You can use the available options of the *Dimensions tab [▸ 887]* to adjust the image view (e.g. use the **Global-Z** slider). The image which is adjusted by the **Dimensions** tab can be selected in the tree view of the **Select Node For Dimensions** tab.

See also

Using the Global-Z slider [▸ 591]

12.20.9.1.8 Aligning images in the Point Alignment Wizard

Prerequisite ✓ You have opened a ZEN Connect project.

1. In the correlative workspace or in the **ZEN Connect** tool, select the image(s) you want to align.
2. In the **ZEN Connect** tool, for the **Alignment** button, select **Point Alignment Wizard** and click on it.
   → The *ZEN Connect Point Alignment Wizard [▸ 610]* opens.
3. In the list on the left, use the [+] button to add as many points as necessary for your point alignment.
   With one point only a translation operation is possible, with two translation and rotation, and three or more points enable all transformations.
4. In the **Algorithm** dropdown list, select the alignment operations you want to perform.
5. Click on **Draw** for the first point.
   → You enter the drawing mode for the first point.
6. In the **Image Window** on the left, click to set the point in your image(s) (**Subject** point).
7. In the **Project Window** on the right, click to set the corresponding location for this point in the project (**Reference** point).
   → Both color makers in the table are green and the first point for the alignment is set successfully.
8. Repeat these three steps for every point you add/need.
9. If you want to redraw a point pair, click on **Redraw** and click to set the new positions in both windows.
10. Click on **Next**.
    → The second step of the wizard opens. It displays a preview of the final alignment result and values for the parameter changes.
11. If you want to change the alignment, click on **Back** to get back to the previous step. Otherwise, click on **Finish** to save the alignment and close the wizard.

You have aligned the selected image(s) in the ZEN Connect project.

12.20.9.2 Zooming to Extent

Prerequisite ✓ You have loaded a **ZEN Connect** project.

1. In the button bar below the **Image View**, click the **Zoom to Extent** icon.

   Alternatively, click on the **Context menu** button and select **Zoom to extent**.

In the **Image View**, the sample holder is centered. All images in the **ZEN Connect** project are displayed.

See also

ZEN Connect Tool [▸ 601]

12.20.9.3 Panning & Zooming

1. In the button bar below the **Image View**, click the **Pan & Zoom** icon.
   → With your mouse you can pan and zoom in and out in the **Image View**.

Shortcut for panning

1. Press the mouse wheel and move the image. Alternatively, press the *Alt* key and left click the image.

You can use panning without having to click the **Pan & Zoom** icon.

See also

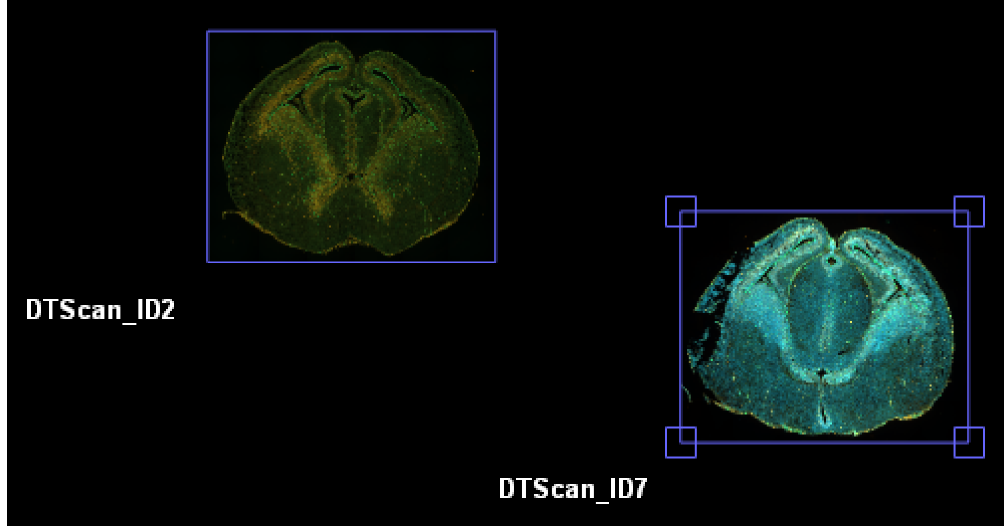
Button bar below Image View [▸ 597]

12.20.9.4 Selecting region

You select a region to later apply the Alignment Mode to the image contained in this region.

Prerequisite ✓ You have loaded a **ZEN Connect** project.

1. In the button bar below the **Image View**, click the **Select Region** icon. Alternatively, press the *CTR* key, and click the desired image in the **Project View**.
   → The image in the selected region is activated.



In the **ZEN Connect** tool, both in the **Project** view and in the **Layers** view, the image within the selected region is highlighted.

12.20.9.5 Toggling the display of region caption and frame

For a better overview, you can toggle the display of the image name and of the frame of images in the **Image view** of your **ZEN Connect** project .

Prerequisite ✓ You have loaded a **ZEN Connect** project.

1. To toggle the region caption, in the button bar below the **Image View**, click the **A** button.

The region caption of the images are displayed or hidden.

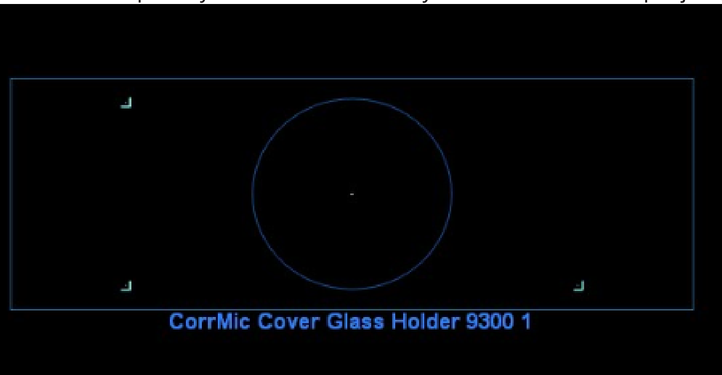
12.20.9.6 Selecting and clearing carrier / holder

The sample is usually mounted on a carrier or directly on a sample holder. Select the appropriate sample holder for your configuration when you configure your project.

We offer specific sample holders and carriers with certain markers, e.g., "L"-markers or others. These CorrMic sample holders are necessary for a Shuttle & Find workflow. Note: If you change the holder/carrier after a S&F calibration, the S&F calibration needs to be redone.

Prerequisite ✓ You have loaded a **ZEN Connect** project.

1. In the button bar below the **Image View**, click the **Select Carrier / Holder** button.
2. Select a template you want to add to your **ZEN Connect** project, and click **OK**.



→ The frame of the selected template is displayed in the **Image View** of your **ZEN Connect** project.

3. To deselect the carrier / holder, click the **Select Carrier / Holder** drop down list and select **Clear / Carrier Holder**.

For information on correlative sample holders, see *Correlative Sample Holders [▸ 496]*.

See also

Select Template Dialog [▸ 594]

12.20.9.7 Grabbing an image

You can create an image from the loaded **ZEN Connect** project.

Prerequisite ✓ You have loaded a **ZEN Connect** project.

1. In the button bar below the **Image View**, select the **Grab Image** button.
   → A new tab opens. In the **Image View**, the grabbed image is displayed.
2. Right-click and save the image to your computer.

You have saved the image. It is not part of your **ZEN Connect** project.

12.20.9.8 Using Regions of Interest in Zen Connect

ZEN Connect offers the functionality to draw and delete Regions of Interest in a project. The regions are shown in the project and listed in the *Regions Tool [**▸ 606]* and they are saved and loaded together with the project.

Drawing a Region of Interest

Prerequisite ✓ You have loaded a **ZEN Connect** project.

1. In the button bar below the **Image View**, click on the button.
2. Draw a rectangular region into your project.

The Region of Interest is displayed in your project.

Deleting a Region of Interest

Prerequisite ✓ You have loaded a **ZEN Connect** project with Regions of Interest.

1. In the **Regions** tool, select your region in the list and click on the button.
   Alternatively, right-click on your region of interest and select **Delete**.

The selected region is deleted.

Renaming a Region of Interest

Prerequisite ✓ You have loaded a **ZEN Connect** project with Regions of Interest.

1. In the **Regions** tool, select your region in the list and click on the button.
2. Enter a name for the selected region and press *Enter*.

The region is renamed according to your input.

Zooming to a Region of Interest

Prerequisite ✓ You have loaded a **ZEN Connect** project with Regions of Interest.

1. In the Image View or the list of the **Regions** tool, right-click on the Region of Interest and select **Zoom To**.

The view zooms to the selected region.

12.20.9.9 Using the Global-Z slider

In ZEN Connect you can use a mode called **Global-Z** if you have a project with at least one z-stack. In this mode, you can change the displayed z plane of all z-stacks in the project with only one slider, the **Global-Z** slider. The range of the slider is comprised of the z values of all stacks in the project. The displayed slice for each stack is always the one closest to the value set by the **Global-Z** slider, as long as the value is within the boundaries of the entire stack. When you use the **Global Z** slider and are beyond the range of a certain z-stack, only a frame is displayed to show where the stack is positioned.
Note: If you have selected a particular z-stack, the **Global-Z** and **Z-Position** sliders are interdependent. Changing the value with one slider updates the other slider as well.

Prerequisite ✓ You have opened a ZEN Connect Project with z-stacks.

1. Activate the **Global-Z** checkbox on the **Dimensions** tab.
2. Use the slider or the input field to set a global value for z.

All the slices closest to the set value are displayed for all z-stacks. Z-stacks which are out of range are illustrated by an empty frame.

12.20.10 ZEN Data Storage

You can store your data on your computer's file system. Additionally, you have the option to save your projects and images in a database called ZEN Data Storage. This makes the information more accessible, as you can search within the database and filter your results. The data storage is an additional product which has to be installed. For more information, refer to the installation guide of ZEN Data Storage.
To activate the access and use of the database, go to **Tools > Modules Manager** and activate **Data Storage Client**. Afterwards please restart the software.

12.20.10.1 Setting up the ZEN Data Storage

After the installation of ZEN Data Storage (for information see the respective installation guide), you have to set up the server in ZEN (blue edition). This setup has to be done once on every machine using the data storage.

Prerequisite
✓ You have installed ZEN Data Storage.
✓ You are logged in as an administrator (if you use ZEN with user management).
✓ In **Tools > Modules Manager**, the **Data Storage Client** is activated.

1. Click on **Tools > Options > Settings**.
2. Under **Document Server Url**, enter the url of your ZEN Data Storage server.
3. Click on **Server Setup**.
4. Click on **Yes** to confirm the message and setup ZEN Data Storage.
   → The server is set up in ZEN and a setup dialog opens.
5. Click on **Close** to exit the setup dialog.
6. Click on **OK** to close the **Tools > Options** dialog.

You have successfully set up your ZEN Data Storage server in ZEN.

See also

Storage Settings Tab [▸ 646]

12.20.10.2 Saving an image to the data storage

You can save any image to the data storage. You can also open images from the data storage, update them, and save the updated image to the data storage.

Prerequisite ✓ You have opened an image.

1. Click on **File > ZEN Data Storage > Save Image**.

The image is saved to the data storage.

12.20.10.3 Saving a ZEN Connect project to the data storage

You can save existing ZEN Connect projects that are currently saved on your computer to the data storage. If you save a project with images, the image information is contained in the **Project View**. You can check it in the **Layers View**.

Optionally, you can create a new project and save it immediately to the data storage.

Prerequisite ✓ You have loaded a ZEN Connect project that is saved to your computer, or you are in the process of creating a new Connect project.

1. Click on **File > ZEN Data Storage > Save ZEN Connect Project**.

The ZEN Connect project is saved to the data storage.

See also

Saving an image to the data storage [592]

12.20.10.4 Opening or deleting an image from the data storage

1. Click on **File > ZEN Data Storage > Open Image**.
   → The **Stored Documents** dialog is displayed.
2. Double-click the image you want to open. Alternatively, select the image and click on **Open**.

The **Stored Documents** closes and the image is loaded in ZEN.

Deleting an image from the data storage

Prerequisite ✓ You have opened the **Stored Documents** dialog.

1. Right-click the image you want to delete, and click on **Delete document**.
   → You are prompted to confirm the operation.
2. Click on **Yes**.

The image is deleted from the data storage.

See also

Stored images in the data storage [607]

12.20.10.5 Opening or deleting a ZEN Connect project from the data storage

Prerequisite

- ✓ You have saved a ZEN Connect project to the data storage. For more information, see *Saving a ZEN Connect project to the data storage [▸ 592]*.
- ✓ If your ZEN Connect project is saved to your computer, open it from there, and save it to the data storage. For more information, see *Loading a ZEN Connect project [▸ 567]*.

1. Click on **File > ZEN Data Storage > Open ZEN Connect project**.
   → A list with stored documents is displayed.
2. Select the ZEN Connect project and click on **Open**.

In the ZEN Connect **Project View**, the current state of the project is displayed. In the **Image View**, the sample holders are marked and previously acquired images are displayed. Note that the project including its images is linked in the data storage. So please take care that these files are not moved or deleted as the links will be broken.

The current stage position is marked with a cross hair.

Deleting a Connect project from the data storage

Prerequisite

- ✓ You have opened the **Stored Documents** dialog.

1. Right-click the project you want to delete and click on **Delete document**.
   → You are prompted to confirm the deleting.
2. Click on **Yes**.

The ZEN Connect project is deleted from the data storage.

See also

ZEN Connect Tool [▸ 601]
Stored Connect projects in the data storage [▸ 606]

12.20.10.6 Configuring the Stored Documents table

In the **Stored Documents** dialog, you select Connect projects or images to open or to delete. You can configure the columns of the table according to your needs.

1. Click on **File > ZEN Data Storage > Open ZEN Connect Project** or **File > ZEN Data Storage > Open Image**.
   → The **Stored Documents** dialog opens.
2. Right-click into the header of the table and activate the columns you want to see in the table.

See also

Stored Connect projects in the data storage [▸ 606]
Stored images in the data storage [▸ 607]

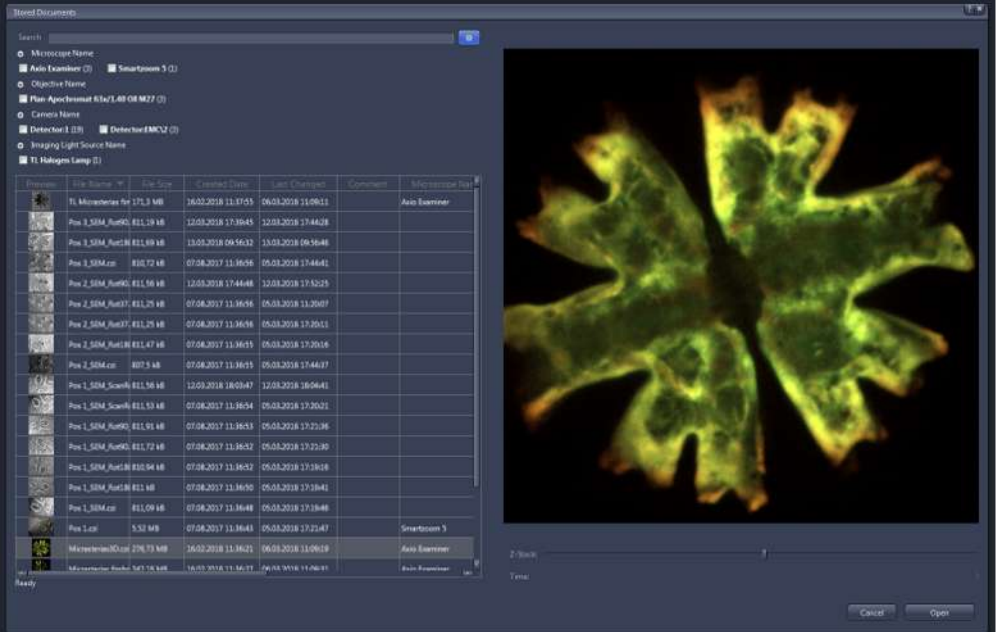
12.20.10.7 Filtering Connect projects and images in the data storage

Prerequisite

- ✓ A ZEN Connect project is available in the data storage.

1. Click on **File > ZEN Data Storage > Open ZEN Connect Project** or **File > ZEN Data Storage > Open Image**.
   → The **Stored Documents** dialog opens.

2. Click on the **Options** button .
   → The search area with filter panel and metadata of the search results is displayed, e.g., **Smartzoom 5**. The number in brackets indicates the amount of search results.



3. To limit the search results, select a term of interest.
   → The available images are displayed accordingly.
4. Optionally, enter a term you are looking for in the **Search** field, e.g., the file name.

The available images are displayed accordingly.

See also

Stored images in the data storage [607]

12.20.11 Functions & Reference

12.20.11.1 Select Template Dialog

In the **Select Template** Dialog, you select carriers and holders.

Parameter	Description
Celldiscoverer	Only available for celldiscoverer application. Shows a list of celldiscoverer sample holders.
Tiles	Shows a list of all generic sample holders.
Correlative	Shows a list with all relevant correlative sample holders.

See also

Selecting and clearing carrier / holder [589]

12.20.11.2 Alignment Tab

The alignment tab is visible, as soon as you enter the alignment mode. For more information, see *Activating the Alignment Process [▸ 576]*.

You can perform Three-Point Alignment:

- Line-up an imported image with reference marks, such as the precision fiducials on a CorrMic Holder.
- Line-up features in an imported image with LM, EM and SEM images of the same features.
- Line-up a session of LM, EM and SEM imagery with previously acquired LM, EM and SEM imagery session.

With the three-point alignment process you can set the position, rotation, and scale of an image or tile. This is used to line the image or tile up with reference marks or other images. Once an image is lined up, it can be used as a reference (road map) to move the stage to control further image acquisition.

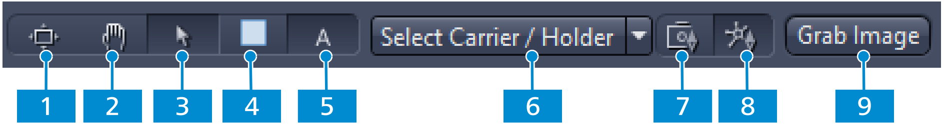
Parameter	Description
Alignment Mode	Sets which data properties you can change during the alignment.
- Translate Only	Moves the item you are aligning in x and y only without changing its size or orientation.
- Translate and Rotate Only	Moves the item in x and y direction and changes its orientation. It does not change the scale of the item you are aligning.
- Translate, Rotate and Scale Only	Moves, reorients, and resizes the item you are aligning. It does not shear it.
- Translate, Rotate, Scale and Shear	Supports full three-point alignment.
- Alignment Handles	Displays alignment handles to rotate and resize the image.
- 3D Alignment	Displays options to align an image in z direction and for three dimensional rotation.
Flip Horizontally	Mirrors the image in the horizontally.
Flip Vertically	Mirrors the image in the vertically.
Flip in Z	Mirrors the image stack in z direction around the middle z-height.
Relative Z Offset	Only visible if **3D Alignment** is selected. Sets the z offset for the selected image.
Step Size	Only visible if **3D Alignment** is selected. Sets the step size for the **Relative Z Offset** input.
Set to current Global-Z	Only visible, if **3D Alignment** is selected and only active if the **Global-Z** slider is activated on the **Dimensions** tab. Sets the z value of the currently selected **Global-Z** as the z value for the center of the z-stack.

Parameter	Description
Apply 3D Rotation	Only visible, if **3D Alignment** is selected. Enables the controls below for the three dimensional rotation of the z-stack.
- Rotation X-Axis	Sets the rotation along the x-axis with the slider or input field. Click on the button to reset the angle.
- Rotation Y-Axis	Sets the rotation along the y-axis with the slider or input field. Click on the button to reset the angle.
- Rotation Z-Axis	Sets the rotation along the z-axis with the slider or input field. Click on the button to reset the angle.
- Angle Step Size	Sets the angle step size for the slider/input fields above.
- View Cube Control	With this cube control, you can rotate the stack interactively. It has a visual representation of the current stack (white box) and the cutting plane which is displayed in the 2D view above.
- Presets	Sets the **View Cube Control** to a predefined position. You can set it to the **Default**, or you can select an orientation from the dropdown of the button to show the **Default**, **Viewer Perspective**, **Left**, or **Right** orientation.
- Manual Control	Controls the orientation of the **View Cube Control**.
Reset	Resets the alignment as it was when you started this alignment operation.
Clear	Resets the alignment to where it was when the data was first acquired or imported.
Finish	Exits from the alignment operation, keeping the alignment you have established.
Cancel	Returns to the alignment as it was when you started, and exits from the alignment operation.

See also

- Aligning image data [576]
- Aligning images in z direction [582]
- Rotating a z-stack [584]

12.20.11.3 Button bar below Image View



Parameter		Description
1	**Zoom To Extent**	Resets the view space of the Image View to be centered on the holder with a field of view (FOV) that includes all visible images in the project. For more information, see *Zooming to Extent [▸ 588]*.
2	**Pan & Zoom**	Activates the mouse for panning around and zooming in and out in the Image View. For more information, see *Panning & Zooming [▸ 588]*.
3	**Select Region**	Selects an image/ a region in the Image View. For more information, see *Selecting region [▸ 588]*.
4	**Create Region of Interest**	Creates a Region of Interest in the Image View. For more information, see *Using Regions of Interest in Zen Connect [▸ 590]*.
5	**Toggle Region Captions**	Hides or displays image names and frames of images in the Image View. For more information, see *Toggling the display of region caption and frame [▸ 589]*.
6	**Select Carrier / Holder**	Opens a dialog to select a carrier or sample holder that matches. For more information, see *Selecting and clearing carrier / holder [▸ 589]*.
7	**Stage Centric View Mode**	Activates a stage centric view mode where the coordinate system of the current session is aligned with the screen and the carrier/sample holder as well as other sessions might be rotated. For more information, see *Toggling view modes [▸ 571]*.
8	**Carrier/ Holder View Mode**	Activates the carrier/holder view mode where the coordinate system of the correlative workspace is aligned with the screen and images of the current session might be rotated. For more information, see *Toggling view modes [▸ 571]*.
9	**Grab Image**	Creates an image of the **ZEN Connect** project. For more information, see *Grabbing an image [▸ 590]*.

12.20.11.4 Configure single image export wizard

With the **Configure single image export** wizard, you configure the parameters of the image you want to export.

Parameter		Description
Color Style		Controls the color format of the export. Select **RGB** (color) or **Intensity** (black and white).
-	RGB	Based on the RGB (Red-Green-Blue) color model. Be aware that color files may be up to three times as large as intensity files.

Parameter	Description
- Intensity	Images are saved in 8-bit format.
Export Format	Selects the format for the exported file. Only the file formats that support the number of pixels you are exporting will be shown. If your export is too large, formats like BMP, JPG and TIFF are not displayed. If you wish to export to one of these formats, you must pick a larger pixel size, or smaller export area for your export.
- Raw image	Exports the image as a raw binary dump of the pixel values. A XML file is also written detailing the image width and height in pixels, the pixel size in microns, the bits per sample and samples per pixel. Raw files are not limited in size.
- CZI image (Single Channel)	Exports the image as a Carl Zeiss Image file. CZI files are not limited in size. The images are exported in a single channel CZI.
- CZI image (Multi-Channel)	Exports the image as a Carl Zeiss Image file. CZI files are not limited in size. The images are exported as a multi-channel CZI. Note that the resulting image might look different when reopened in **ZEN** than it does in **ZEN Connect**.
- Tif image	Exports the image as a standard TIFF file. TIFF files are limited in size.
- Tif tiles	With this option, the export is in TIFF format, but broken into 2K x 2K tiles saved as individual TIFFs. An XML file is written listing the file names of the tiles and their positions. This export option is unlimited in size, but designed for someone who is writing scripts to import the data into image processing applications or similar.
- Bitmap image	Exports the image as a standard Windows Bitmap. Bitmap files are limited in size.
- Jpg image	Exports the image as a standard JPEG file. JPEG files are limited in size.
Show Data Bar	Burns the currently configured data bar into the exported image. Activate the **Show Data Bar** checkbox.
Show Region Caption	Controls if image names and frames are shown in the exported image.
Rotation	Rotates the view to the desired orientation. Drag the slider, or in the text field, type in the value.
Pixel Size	Sets the pixel size of the export. Type the pixel size in the **Pixel Size** field. The smaller the pixel size, the more disk space your export will take. Click the **1:1** button to reset the pixel size to the native resolution.
Width (px)	Width value to directly alter the export pixel counts and export area (the pixel size is unchanged).
Height (px)	Height value to directly alter the export pixel counts and export area (the pixel size is unchanged).
Width (µm)	The full width of the export area in µm.
Height (µm)	The full height of the export area in µm.
Approx. Data Size	The actual file size after export may be less than the listed data size based on compression in some file formats.

Parameter	Description
Export Data	Starts the export with your settings.
Finish	Saves the changes and closes the wizard.
Cancel	Closes the wizard without saving the changes.

For more information on exporting single images, see *Exporting single image data [▸ 574]*.

12.20.11.5 Field of View Width dialog

Parameter	Description
FOV Width	Displays the current width of the field of view and allows you to enter a value.
OK	Sets the width to the entered value.
Cancel	Closes the dialog box without setting the field of view.

12.20.11.6 Pixel Size dialog

Parameter	Description
Pixel Size	Displays the current pixel size and allows you to enter a value.
OK	Sets the pixel size to the entered value.
Cancel	Closes the dialog box without setting the pixel size.

12.20.11.7 Video Export wizard

Parameter	Description
Burn in Data Bar	Burns the currently configured data bar into the exported video.
Show Region Caption	Controls if the image names are shown in the exported video.
Show Region Outline	Controls if the image frames are shown in the exported video.
Export Resolution	Sets the resolution and format for the video export. Resolutions available in the drop down list: ▪ 320 x 240 (4:3) ▪ 428 x 240 (16:9) ▪ 640 x 480 (4:3) (=default value) ▪ 854 x 480 (16:9) ▪ 960 x 720 (4:3) ▪ 1280 x 720 (16:9)

Parameter	Description
	▪ 1440 x 1080 (4:3) ▪ 1920 x 1080 (16:9)
Zoom to Extent	Places the image to the center of the preview area.
Rotation	You can use the slider or the input field to rotate the view.
Start Delay	Sets the delay at the start of the video. The default setting is 1,0 seconds.
Key Frames	Lists the key frames, including the data of the position and FOV.
Move Up	Moves the selected key frame up in the list.
Move Down	Moves the selected key frame down in the list.
Delete key frame	Deletes the selected key frame.
Go to key frame	Displays the selected key frame.
Reset key frame to current view	Sets the values of the selected key frame to the current view.
Options	
– Load Export Key Frames	Loads stored key frames.
– Save Export Key Frames	Saves the key frames in XML format.
Transit To	Sets the transition time for zooming to a selected key frame. The default setting is 3,5 seconds.
Pause At	Sets the time to stay at the selected key frame. Default setting is 0,5 seconds.
Return to first at end	Returns to the first key frame at the end of the video.
Add current view as key frame	Adds the current view as a key frame.
Preview export	Displays a real-time preview of the video.
Export Data	Exports and saves the video.
Finish	Saves the changes and closes the wizard.
Cancel	Cancels the video export.

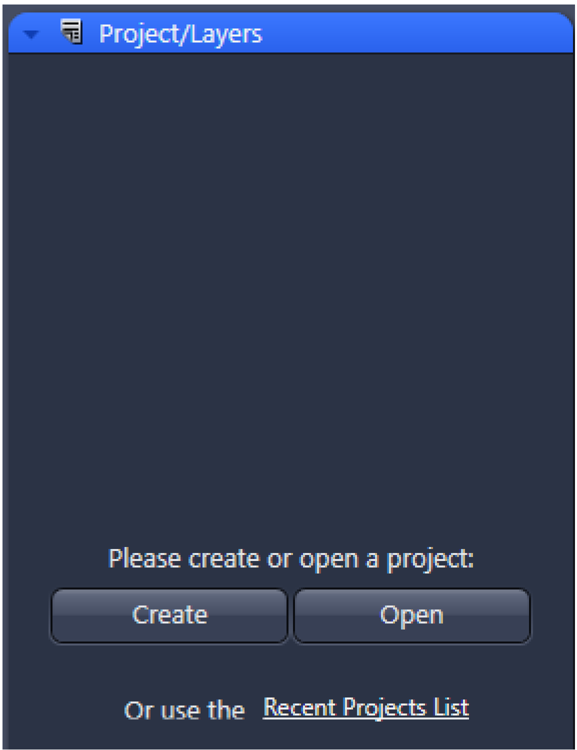
See also

Exporting a Zen Connect project as a video [574]

12.20.11.8 ZEN Connect Tool

The **ZEN Connect** tool provides a layers view and a tree view of image data that you have acquired for the **ZEN Connect** project. Every image that you have acquired for the **ZEN Connect** project is listed. As you acquire or import more image data, the new image data will be listed in the views.

The **ZEN Connect** tool offers different options to open a **ZEN Connect** project or to create a new **ZEN Connect** project.



Alternatively, you can create a new **ZEN Connect** project via **File > New Document**. For more information, see *New Document Dialog [▸ 616]*.

The **ZEN Connect** tool displays the following:

- Images that have been acquired for the **ZEN Connect** project.
- Images that have been imported for the **ZEN Connect** project.
- Position of the image in the project or the layers.

12.20.11.8.1 ZEN Connect Project tree view

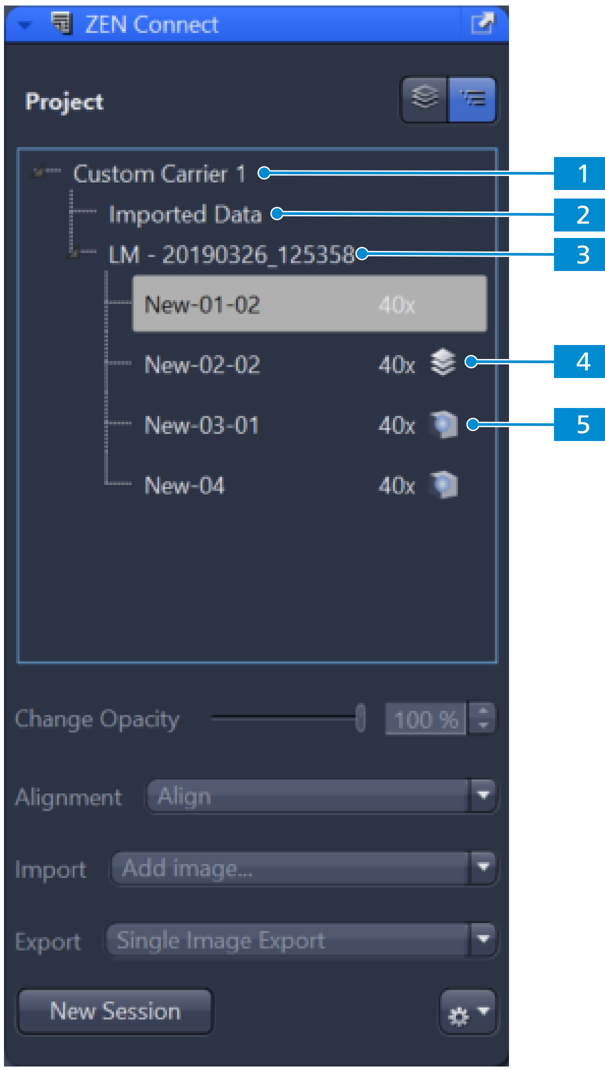


*Fig. 47: Tree view: **ZEN Connect** project (Example)*

1 Sample holder
Here: **Custom Carrier 1**

2 **Imported Data**
Subordinated, the data added to the **ZEN Connect** project is displayed. These images are not acquired within the Connect project.

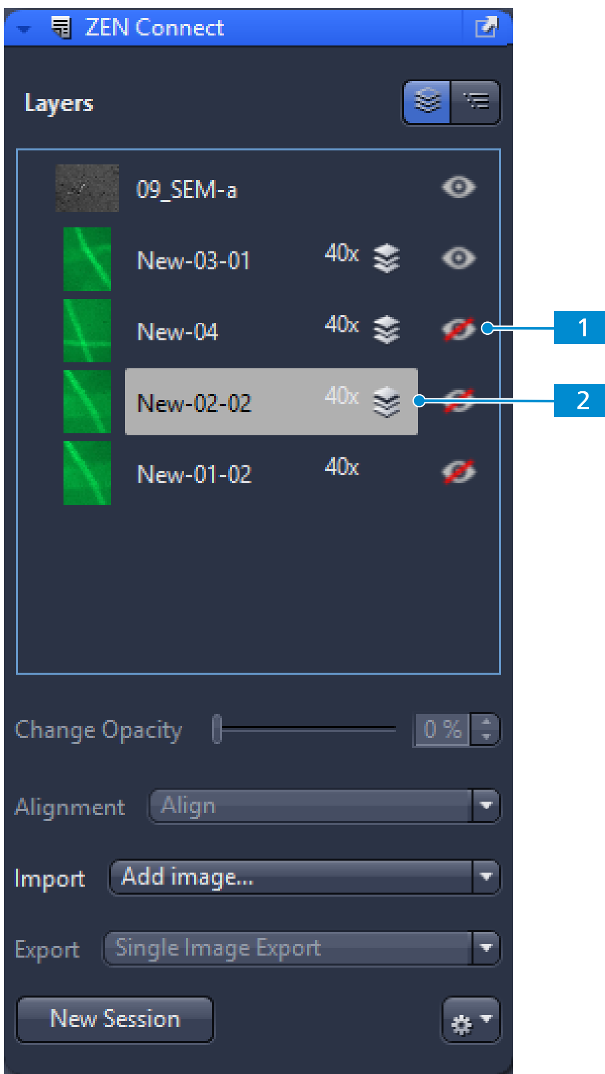
3 On top, the session node is displayed. It contains the following information. Subordinated, the acquired images are listed.

Here: **LM** (Microscope)
Here: **20190326** (Date <yyyymmdd>)
Here: **125358** (Time <hhmmss>)
Here: **New-01-02** (Image taken in this session)

4 Image Information
Displays the objective magnification with which an image was acquired (here: **40x**). Additionally, the icon is displayed for a z-stack image.

5 Z-Stacks which are opened in the *ZEN Connect 3D view [▸ 564]* are displayed with another icon in the tree view.

12.20.11.8.2 ZEN Connect Project Layer view



Images in your **ZEN Connect** project are displayed in the **Image View** according to its position in the **Layers View**. With drag & drop, you can move images over and under other images. You can also hide them completely 1. For more information, see *Moving or hiding images [▸ 570]*. Additionally, you can see the objective magnification with which the image was acquired and if an image is a z-stack 2.

12.20.11.8.3 Button bar

Parameter	Description
Change Opacity	Changes the opacity of layers. A layer's overall opacity determines to what degree it obscures or reveals the layer beneath it. A layer with 1% opacity appears nearly transparent, whereas one with 100% opacity appears completely opaque.
Alignment	Only available if you have selected one or more images or a session. Starts the currently selected alignment operation for the selected image(s) or session. Click on the arrow button to select an alignment.
– Align	Activates the alignment process for the selected data. For more information, see *Activating the Alignment Process [▸ 576]*.

Parameter	Description
– Align system	Activates the alignment process for the current microscope session. For more information, see *Activating the Alignment Process [▸ 576]*.
– Manual Alignment Wizard	Opens the *ZEN Connect Manual Alignment Wizard [▸ 608]* for manual alignment. For more information, see also *Aligning images in the Manual Alignment Wizard [▸ 586]*.
– Point Alignment Wizard	Opens the *ZEN Connect Point Alignment Wizard [▸ 610]*. For more information, see also *Aligning images in the Point Alignment Wizard [▸ 587]*.
Import	Starts the currently selected operation to add and/or import an image. Click on the arrow button to select an import operation. For more information, see also *Importing Data [▸ 572]*.
– Add image	Opens a explorer to add an image. For more information, see *Adding an image to the ZEN Connect project [▸ 567]*.
– Add image from storage	Opens a window to add an image from the ZEN Data Storage. For more information, see *Adding an image to the ZEN Connect project [▸ 567]*.
– Import non-image data	Opens the file browser to select non-image data for an import. For more information, see also *Importing non image data [▸ 574]*.
– Import SmartFIB z-stack	Imports a SmartFIB z-stack. For more information, see *Importing a SmartFIB stack into ZEN Connect [▸ 573]*.
– Add dataset	Adds a dataset. For more information, see *Adding datasets when adding images [▸ 569]*.
Export	Starts the currently selected operation to export an image. Click on the arrow button to select an export operation.
– Single Image Export	Exports the selected data in one single image. For more information, see *Exporting single image data [▸ 574]*.
– Video Export	Exports the selected data as a video. For more information, see *Exporting a Zen Connect project as a video [▸ 574]*.
– Export Project	Opens a file browser to export your current ZEN Connect project. For more information, see also *Exporting a ZEN Connect project from the data storage [▸ 575]*.
New Session	Creates a new session. For more information, see *Starting a new session [▸ 571]*.
Context menu	
– Zoom to	Zooms one or more selected images into the center of the **Image View**. For more information, see *Zooming images [▸ 569]*.

Parameter	Description
– Zoom to 100%	Zooms to the selected image in the **Image View** and displays it at 100% scale. For more information, see *Zooming images [▸ 569]*.
– Show/Hide	Toggles the image/session between being shown or being hidden. For more information to hide images, see *Moving or hiding images [▸ 570]*.
– Open image(s) in ZEN	Opens the selected image(s) in ZEN. For more information, see *Opening images in ZEN [▸ 570]*.
– Open Attachment	Only available for non-image data. Opens the attached file in the respective application.
– Show in Explorer	Locates an image or file on your computer. For more information, see *Showing an image in the Explorer [▸ 570]*.
– Remove Data	Removes the selected image from the **ZEN Connect** project, but does not delete it from the computer. For more information, see *Removing images from the ZEN Connect project [▸ 569]*.
– Delete	Only visible for non-image data. Deletes the file from the project and from the project folder. If the file was added as a link, only the link in the project is deleted.
– Rename Data	Renames an image. For more information, see *Renaming images in a ZEN Connect project [▸ 570]*.
– Align Data	Aligns data of the two selected images. For more information, see *Aligning image data [▸ 576]*.
– Single Image Export	Exports the selected data in one single image. For more information, see *Exporting single image data [▸ 574]*.
– Video Export	Exports the selected data as a video. For more information, see *Exporting a Zen Connect project as a video [▸ 574]*.
– Add image	Opens a explorer to add an image. For more information, see *Adding an image to the ZEN Connect project [▸ 567]*.
– Add image from storage	Opens a window to add an image from the ZEN Data Storage. For more information, see *Adding an image to the ZEN Connect project [▸ 567]*.
– Import SmartFIB z-stack	Imports a SmartFIB z-stack. For more information, see *Importing a SmartFIB stack into ZEN Connect [▸ 573]*.
– Show in 3D	Only available if two images are selected in the tree view. Opens the two images in the ZEN Connect 3D viewer. For more information, see *Opening images in ZEN Connect 3D view [▸ 565]*.
– New Session	Starts a new session. The images acquired next will be subordinated below a new session node. For more Information, see *Starting a new session [▸ 571]*.

Parameter	Description
– Clear alignment	Resets the alignment and places the selected image at its initial position.
– Zoom to extent	Resets the view space of the **Image View** to be centered on the holder with a field of view (FOV) that includes all visible images in the project. For more information, see *Zooming to Extent [▸ 588]*.
– Export Project	Opens a file browser to export your current ZEN Connect project. For more information, see also *Exporting a ZEN Connect project from the data storage [▸ 575]*.
– Import non-image data	Opens the file browser to select non-image data for an import. For more information, see also *Importing non image data [▸ 574]*.

12.20.11.9 Regions Tool

This tool displays a list of Regions of Interest which are drawn into a **ZEN Connect** project.

Parameter	Description
Delete	Deletes the Region of Interest selected in the list.
Rename	Allows the user to rename the Region of Interest which is currently selected in the list.

See also

Using Regions of Interest in Zen Connect [▸ 590]

12.20.11.10 Stored Connect projects in the data storage

You can configure the table according to your needs. For more information, see *Configuring the Stored Documents table [▸ 593]*. You can also filter data of stored documents. For more information, see *Filtering Connect projects and images in the data storage [▸ 593]*.

Parameter	Description
Search	Searches the database for the term entered in the text field.
File name	Displays the name of the ZEN Connect project. You can sort the file names alphabetically.
File Size	Displays the file size.
Created Date	Displays the creation timestamp.
Last Access	Displays at what date the image was last opened.
Comment	Displays a comment.
Cancel	Closes the dialog.

Parameter	Description
Open	Opens the Connect project. For more information, see *Opening or deleting a ZEN Connect project from the data storage [▸ 593]*.

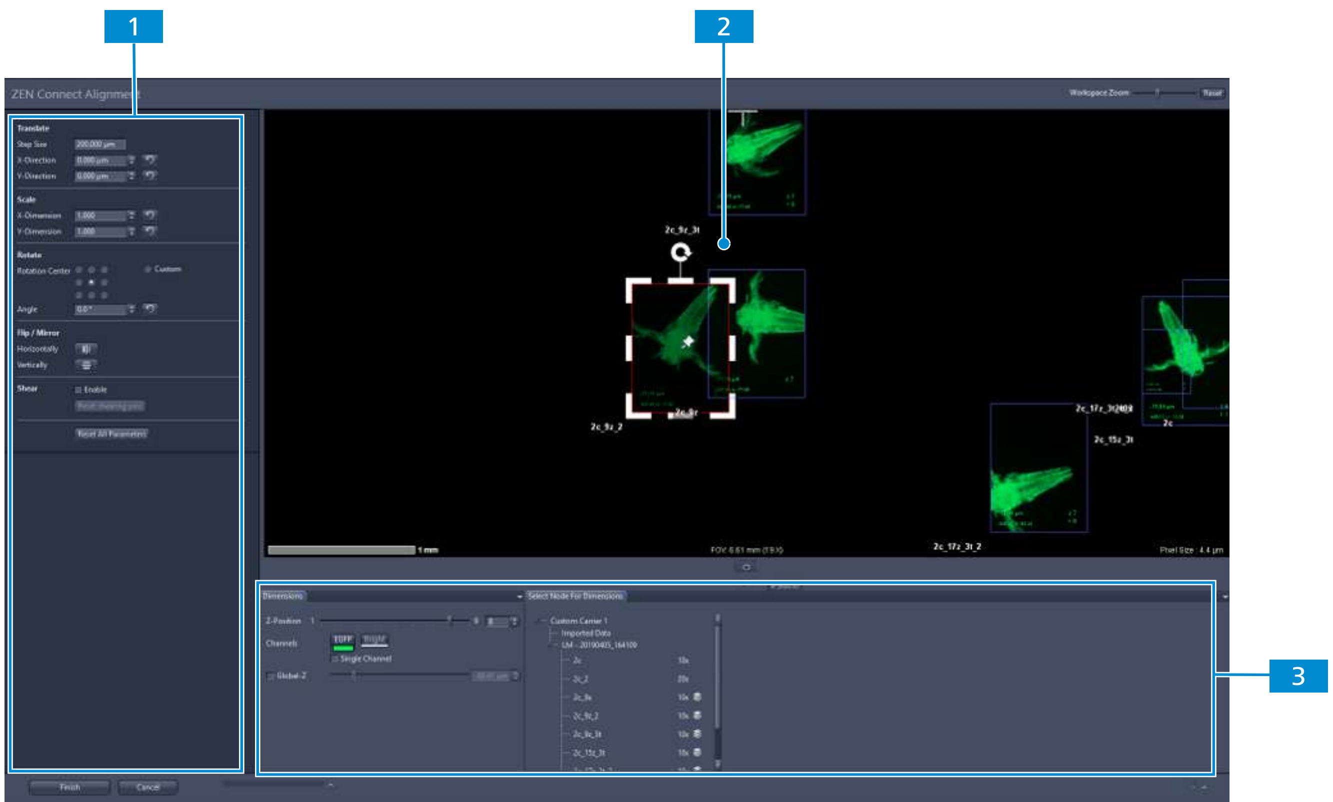
12.20.11.11 Stored images in the data storage

Double-click to open the image. For more information, see *Opening or deleting an image from the data storage [▸ 592]*.
You can configure the table according to your needs. For more information, see *Configuring the Stored Documents table [▸ 593]*. You can also filter data of stored documents. For more information, see *Filtering Connect projects and images in the data storage [▸ 593]*.

Parameter	Description
Search	Searches the database for the term entered in the text field.
Options	Displays a filter panel with metadata.
Preview	Displays the preview of the image.
File Name	Displays the file name and the format of the image. You can sort the file names alphabetically.
Original File Name	Displays the original file name in case you have uploaded the image as a third party image with the ZEN Data Storage Uploader.
File Size	Displays the file size.
Software Application	Displays with which software application the image was acquired.
Software Application Version	Displays the version of the software application.
Created Data	Displays the creation day.
Last Change	Displays at what date the image was last changed.
Comment	Displays a comment.
Microscope Name	Displays the name of the microscope.
System Name	Displays the system name.
Objective Name	Displays the name of the objective.
Objective Magnification	Displays the objective magnification.
Reflector	Displays the reflector.
Channel Name	Displays the name of the channel.
Camera Name	Displays the name of the camera.
Scaling	Displays the scaling.

Parameter	Description
Imaging Light Source Name	Displays the name of the imaging light source.
Z-Slider	If the image contains a z-stack, with the slider you can select a single z-slice.
T-Slider	If a time series is contained in the image, with the T-slider you can se-lect a certain point of time.

12.20.11.12 ZEN Connect Manual Alignment Wizard



1 **Alignment Parameter**
Control parameters to align the respective image in the project. For more information, see *Alignment Parameter section [▸ 609]*.

2 **Image View**
Displays the images of the project and allows alignment of images.

3 **View Options**
Here you have the general options of the *Dimensions tab [▸ 887]*. The options are always those of the image selected in the **Select Node For Dimensions** tab.

See also

Aligning images in the Manual Alignment Wizard [▸ 586]

12.20.11.12.1 Alignment Parameter section

Parameter	Description
Translate	
– Step Size	Sets the step size for the translation in x and y.
– X-Direction	Sets the translation in x direction. The reset button resets the value to the default.
– Y-Direction	Sets the translation in y direction. The reset button resets the value to the default.
Scale	
– X-Direction	Sets the scaling factor in x direction. The reset button resets the value to the default.
– Y-Direction	Sets the scaling factor in y direction. The reset button resets the value to the default.
Rotate	
– Rotation Center	Defines the rotation center around which the image is rotated. It is indicated in the Image View with a pin.
– Angle	Sets the rotation angle. The reset button resets the value to the default.
– Custom	You can set the pin in the image to your custom rotation center.
Flip/Mirror	
– Horizontally	Mirrors the image in horizontal direction.
– Vertically	Mirrors the image in the vertical direction.
Shear	
– Enable	**Activated:** Activates the shearing mode and displays the three shearing pins in the image.
– Reset Shearing Pins	Resets the pins to the default location.
Reset All Parameters	Resets all alignment parameters in the wizard to the default values.

Parameter	Description
Finish	Saves the changes and closes the wizard.
Cancel	Closes the wizard without saving.

See also

Aligning images in the Manual Alignment Wizard [586]

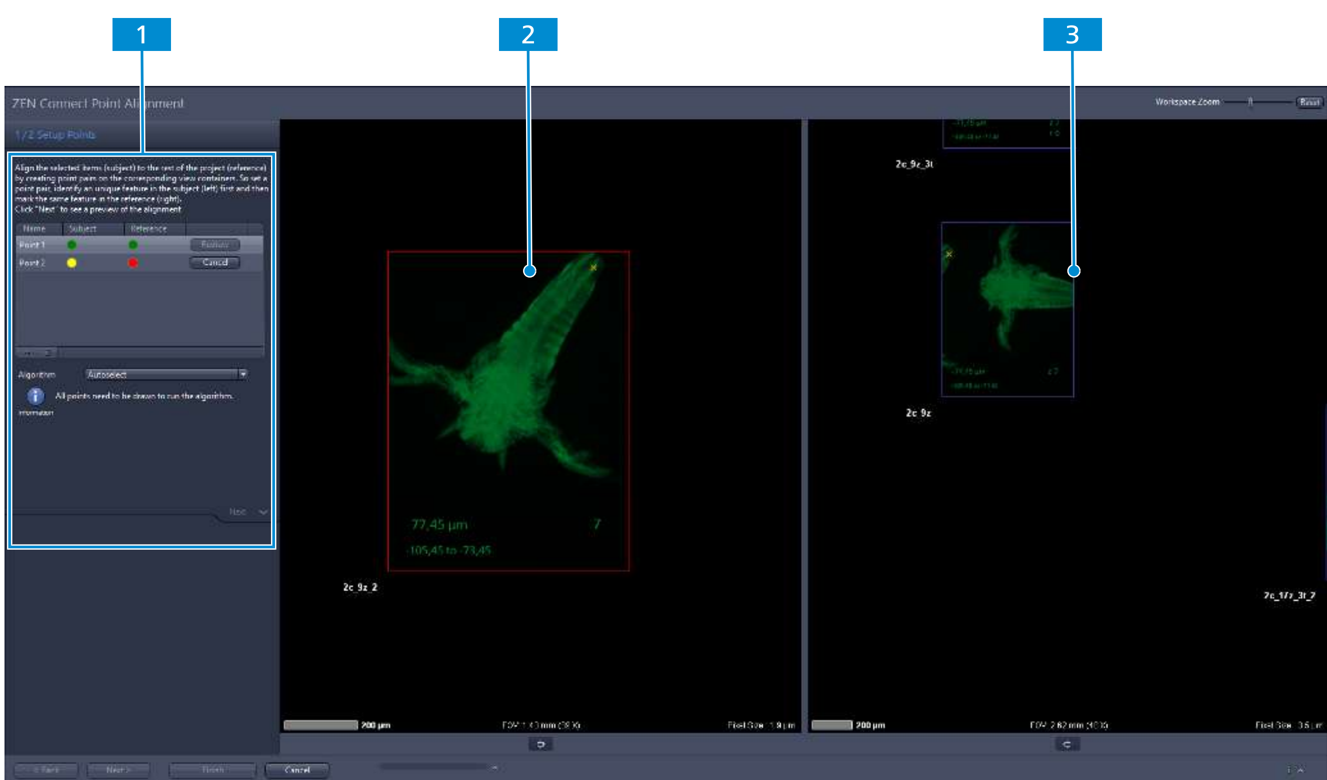
12.20.11.13 ZEN Connect Point Alignment Wizard

This wizard guides you through a three-point alignment of the image in your ZEN Connect project.

See also

Aligning images in the Point Alignment Wizard [▸ 587]

12.20.11.13.1 Step 1: Setup Points



1 **Point Alignment Options**
Options to configure your point alignment. For more information, see *Point Alignment Options [▸ 610]*.

2 **Image Window**
Displays the image(s) you are aligning. Area where you set the reference points.

3 **Project Window**
Displays all the images of the project except the one you are aligning. Area where you set the subject points.

See also

Aligning images in the Point Alignment Wizard [▸ 587]

12.20.11.13.1.1 Point Alignment Options

Parameter	Description
Point list	
– Name	Displays the name of the point.

Parameter	Description
– Subject	Displays the status of the subject point with a color. ▪ Yellow: You are in drawing mode and have not yet set the subject point in the Image Window. ▪ Green: You have set the subject point.
– Reference	Displays the status of the reference point with a color. ▪ Red: You have not yet set the subject point and cannot set the subject point. ▪ Yellow: You are in drawing mode and have not yet set the reference point in the Project Window. ▪ Green: You have set the reference point.
– Draw	Only visible if no points have been drawn for the current point entry. Enters the drawing mode for the respective point.
– Cancel	Only visible if you are in drawing mode. Cancels the drawing of points for the current point.
– Redraw	Only visible if you have already drawn the reference and subject point for this entry. Reenters the drawing mode to redraw the points for this entry.
– Add	Adds another point entry in the list.
– Delete	Deletes the currently selected list entry and removes all drawn points of the entry.
Algorithm	Selects the algorithm for alignment. The algorithm is preselected based on the number of positioned points.
– Autoselect	Automatically selects the algorithm suitable for the drawn points.
– Translation	Moves the item you are aligning in x and y only, without changing its size or orientation.
– Translation and Rotation	Moves the item in x and y direction and changes its orientation. It does not change the scale of the item you are aligning.
– Translation and Scale	Moves and resizes the item you are aligning.
– Allow all transformations	Supports all possible transformations.

Parameter	Description
Next	Moves on to the next step of the wizard.
Finish	Saves the changes and closes the wizard.
Cancel	Closes the wizard without saving.

See also

Aligning images in the Point Alignment Wizard [▸ 587]

12.20.11.13.2 Step 2: Preview

This step displays a preview of the finished alignment and the parameter values of each alignment.

Parameter	Description
Algorithm Result	Displays the resulting alignment changes.
Translation	Displays the resulting translation in **X-Direction** and **Y-Direction**.
Rotation	Displays the resulting rotation angle around the z-axis.
Scaling	Displays the resulting scaling factor for the **X-Dimension** and **Y-Dimension**.
Back	Moves to the previous step of the wizard.
Finish	Saves the changes and closes the wizard.
Cancel	Closes the wizard without saving.

13 Software Functions & Reference

13.1 Menus

13.1.1 File Menu

Menu item	Description	Short cut
New Document	Opens the New Documents selection dialog. You can create new images or new tables. For more information, see *New Document [616]*.	*Ctrl + Shift+N*
New Image	Open an empty image container in the center screen area into which you can snap an image, for example.	*Ctrl+N*
Open...	Opens the **Open Document** dialog window. Here you can select the file you want to open.	*Ctrl+O*
Save	Saves the selected file.	*Ctrl+S*
Save As CZI	Saves the selected file under a new name. In case of an image only **.czi** file format can be used.	*Ctrl +Shift+S*
Save As with Options...	Saves the selected file under a new name. Advanced options can be selected: **File type**: czi, jpeg, jpg, png, tif, tiff, bmp, gif, wmp, wdp **Compression** (only for **czi** and **jpg/jpeg**): ▪ **Original**: The image keeps the compression of the original image. ▪ **Uncompressed**: The image is saved without compression. ▪ **Compressed (JPEG XR)**: An uncompressed image will be compressed with the selected quality. A compressed image keeps the compression. ▪ **Force Compression (JPEG XR)**: A compressed image will be decompressed and compressed with the selected quality. **Zoom Level** (only for pyramid images): Different zoom levels can be selected, depending on the image pyramid. **Set as default**: Sets the selected options as default saving options.	
Rename	Opens the **Rename** dialog window. Enter a new name for the file. Confirm the entry with **Yes**.	
Delete	Deletes the selected file.	
Export/Import	▪ **Export** Opens the image processing function *Image Export [207]* to export an image.	*Ctrl+6*

Menu item	Description	Short cut
	▪ **Movie Export** Opens the image processing function *Movie Export [▸ 195]* to export images in the form of film sequences. ▪ **OME TIFF Export** Opens the image processing function *OME TIFF Export [▸ 200]* to export images into OME TIFF format. ▪ **ZVI Export** Opens the image processing function *ZVI Export [▸ 203]* to export images into ZVI format. ▪ **MRC Export** Exports image data into the MRC format, which is compatible to SerialEM. It also creates a NAV file, which stores positions of interest. For more information, see *Exporting data for SerialEM [▸ 575]*. ▪ **Import** Opens the image processing function *Image Import [▸ 204]* to create a multidimensional image (multichannel, Z-stack, time lapse, tile, position image) from individual images. ▪ **BioFormats Import** Imports third-party images using BioFormats as an integrated library for reading and writing life sciences image file formats. For more information, see *Importing Third-party images [▸ 572]*. ▪ **Import TXM** Opens the image processing function *Import TXM [▸ 206]* to import a txm file to ZEN.	
Send to arivis Vision 4D	This function is only available, if arivis Vision 4D is installed and has a valid license. When using this command, Vision 4D opens and the current image is imported into Vision 4D. The current display settings in ZEN are preserved as much as possible. **Note:** special CZI image types such as ApoTome raw images, Airyscan raw images, or some other image types are not always supported in Vision 4D. In these cases convert them into normal processed images before sending them to Vision 4D for further analysis.	
Add to ZEN Connect Project	Adds the image to the currently opened ZEN Connect project.	
Send to ZEN Black	The software installation includes a copy of **ZEN Black**. In some cases it might be necessary to transfer images from the software to **ZEN Black** to make use of special functions found only in **ZEN Black**.	
Close	Closes the selected file.	*Ctrl+F4*
Save All	Saves all open files.	
Open File Browser	Opens the file browser in the **Center Screen Area**.	*Ctrl+F*

Menu item	Description	Short cut
Open Containing Folder	Opens the folder in which the selected file is located.	
ZEN Data Storage	Only available if you have a connection to ZEN Data Storage and the module is activated in **Tools > Options**. ▪ **Open ZEN Connect Project** Displays a dialog to open a Connect project from data storage. ▪ **Save ZEN Connect Project** Saves the current Connect project to the data storage. ▪ **Open Image** Displays a dialog to open an image from data storage. ▪ **Save Image** Saves the current image to the data storage.	
Recent Files...	Opens the **Recent Files** dialog window. The **Recent Files** dialog displays the files you have used previously, separated according to file type.	*Ctrl+R*
Recently Opened Files	Opens a list containing the file paths of recently opened files.	
Print Preview	Opens the *Print Preview [▸ 617]* dialog for the selected file.	*Ctrl+F2*
Login	Opens the **Login** dialog window.	
Tiles	Opens a submenu with functions associated with the tiles tool. Here, parameters can be imported and exported. ▪ **Export Tiles Experiment** Opens a dialog to export the current Tiles experiment either to an XML or a CSV file. ▪ **Import Tiles Experiment** Opens a dialog to import a Tiles experiment either from an XML or a CSV file. ▪ **Import Stage Marks** Imports the existing positions from the **Stage** Tool (Marks) as single positions in the Tiles experiment. ▪ **Import Preview Image** Imports an existing image document as a preview image into the **Navigation/Tiles** view. This function is also available with right mouse-click in the **Tiles - Advanced Setup** view. ▪ **Save Preview Images** Saves existing preview/snap images into a separate image document.	

Menu item	Description	Short cut
	▪ **Extract Sample Carrier Template** Opens a dialog to extract the current sample carrier template from the Tiles experiment as a new template with the calibrated dimensions.	
Exit	Exits the software.	*Alt+F4*

See also

Image Export [▸ 207]

13.1.1.1 New Document Dialog

Here you can create different types of new, empty documents (images, tables, and ZEN Connect projects).

Select the desired document type and click on **OK**. The image or table will be generated and opened in the current workspace.

Parameter	Description
Images	Creates a new, empty image file (*.czi).
Tables	Creates a new table (*.czt). The following elements are only visible if you have clicked on the **Tables** button:
- Document name	Here you can enter the name of the new table-document.
- Columns	Enter the number of columns that you want the new table to have in the input field.
- Rows	Enter the number of rows that you want the new table to have in the input field.
- Column Name	Here you can enter the title of the column.
- Column type	Here you can select the desired data type of a column. The following types are available: ▪ String ▪ Integer ▪ Real
- Default Value	Here you can enter a default value that you want the column cell to contain.
ZEN Connect Project	Creates a ZEN Connect project. For more information, see *Creating a ZEN Connect project [▸ 565]*.

13.1.1.2 Print Preview Dialog

Parameter	Description
Printer	Here you can select the printer that you want to use.
Properties	Opens a dialog window containing the printer properties. Here you can configure advanced settings. This dialog window is dependent on the printer.
Format	Here you can select the page format e.g. A0 to A6, Letter or A4 Register.
Width	Displays the width of the page according to the chosen format.
Height	Displays the height of the page according to the chosen format.
All pages	**Activated:** Prints all pages of the report.
Selected Pages	**Activated:** Prints a certain number of pages of the report. In the input field to the right, enter the pages that you want to print. **Example:**The entry **1-3; 5** prints pages 1,2,3 and 5.
Pages per sheet	Here you can select how many pages you want to print on one sheet.
Number of copies	Here you can enter the number of copies that you want to print.
Collated	Only active if you have chosen to print several copies. **Activated:** Sorts the pages of each copy printed.
Auto fit	**Activated:** Adjusts the size of the report or image to the size of the page.
Scale pages	**Activated:** Adjusts the size of the report or image to the factor set in the input field to the right. Here you can set the desired enlargement/reduction factor for the report or image. A factor of 100% corresponds to the **Auto fit** option.
Print	Prints the report using the options set.
Down	Displays the next page.
Up	Displays the previous page.
Auto fit	Here you can select the zoom factor with which the page view is displayed in this dialog.

13.1.2 Edit Menu

Menu item	Description	Short cut
Undo	Undoes the last action.	*Ctrl+Z*
Redo	Redoes the last action.	*Ctrl+Y*
Cut	Cuts the selected graphic element out of the image.	*Ctrl+X* *Shift +Del*

Menu item	Description	Short cut
Copy	Copies the selected graphic element.	*Ctrl+C* *Ctrl+Ins*
Paste	Inserts the copied graphic element into the image.	*Ctrl+V* *Shift+Ins*
Delete	Deletes the selected element.	*Del*
Select All	Selects all graphic elements drawn into the image.	*Ctrl+A*
Display	Here you can manage image display settings. Functions include copy, paste, export or import of the display settings.	
ROI (Region of Interest) | Draw Region of Interest	Draw a certain rectangular region that is of particular interest to you into the image. The ROI is displayed with red boundaries. You can draw several regions into an image.	*Ctrl+U*
ROI (Region of Interest) | Draw Rotatable Region of Interest	Draw a certain rectangular region that is of particular interest to you into the image. This region can be rotated and is displayed with yellow boundaries. You can draw several regions into an image.	*Ctrl+Shift+R*
ROI (Region of Interest) | Create Subset Images from ROI	Creates new image documents from the selection regions you have drawn in. All dimensions of the image are taken into account here. This function works for both the non rotatable and rotatable ROIs (the red and the yellow regions).	*Ctrl+Shift+C*
Create Image from View	Creates an image from the current view.	

13.1.3 View Menu

Menu item	Description
Zoom	Here you can configure various zoom settings.
Player	Here you can navigate through a Z-stack or a time series image.
Text View	Displays the text name of a file in the **Document bar**.
Small Thumbnail View	Displays a small preview image and the name of a file in the **Document bar**.
Large Thumbnail View	Displays a large preview image and the name of a file in the **Document bar**.
1 Container	Displays one image container in the **Center Screen Area**.
2 Containers	Displays two image containers in the **Center Screen Area**.
3 Containers	Displays three image containers in the **Center Screen Area**.
Automatic Container Layout	Uses the predefined container layout.

Menu item	Description
Shared View Controls	General and specific view controls are shared for all containers and are active for the currently selected image container.
Separate View Controls	Each container has its own separate general and specific view controls that become active when the associated image container is selected.
Show All (Global)	Activates the **Show All** mode in every tool.
Show Macro Environment	Activates the *Macro Tool [▸ 804]* in the **Right Tool Area**. If you have licensed the **Macro Environment** module, the **Macro** menu appears in the **Menu bar**. The Macro Environment is deactivated by default.

13.1.4 Acquisition Menu

Menu item	Description	Short cut
Locate Snap	Acquires a single image with the active camera.	*F2*
Locate Live	Shows a live image from the active camera in the **Center Screen Area**. On the **Locate** tab the **Live** mode is activated.	*Shift+F2*
Acquisition Snap	Executes only if you have defined at least one channel on **Acquisition** tab in the **Channels** tool. Acquires and displays all the defined channels as an overlay.	
Acquisition Live	Executes only if you have defined a channel on **Acquisition** tab in the **Channels** tool. Uses the currently selected channel for a live image.	
Set Exposure	Executes only if you have defined a channel on **Acquisition** tab in the **Channels** tool. Executes an exposure time calculation for the active camera.	
Set White Balance	Executes a white balance measurement.	*Alt+W*
Find Focus	Executes the Software Autofocus Running. On the **Acquisition** tab the **Find Focus** mode is activated.	
Start Experiment	Only active, if you have configured an experiment on **Acquisition** tab. Starts a defined experiment.	

Menu item	Description	Short cut
Stop Experiment	Only active, if you have started an experiment on **Acquisition** tab. Stops a running experiment.	
Pause Experiment	Only active, if you have started an experiment on **Acquisition** tab. Pauses a running experiment.	
Continue Experiment	Only active, if you have paused an experiment on **Acquisition** tab. Continues the paused experiment.	
Dual Camera Calibration Wizard…	Only visible if a dual camera configuration is active. Starts **Dual Camera Calibration Wizard.**	
ApoTome Phase Calibration Wizard…	Only visible if a ApoTome configuration is active. Starts **ApoTome Phase Calibration Wizard.**	
ApoTome Focus Calibration Wizard…	Only visible if a ApoTome configuration is active. Starts **ApoTome Focus Calibration Wizard.**	

13.1.5 Graphics Menu

Menu item	Description	Short cut
Select	Starts the selection mode.	*Alt+F1*
Draw Region of Interest	Allows you to draw in a region of interest (ROI).	*Crtl+U*
Text	Allows you to add a text field to the image.	
Scale bar	Allows you to add a scale bar to the image.	
Line	Allows you to add a line to the image.	
Arrow	Allows you to add an arrow to the image.	
Rectangle (aligned)	Allows you to add an aligned rectangle to the image.	*Alt+F2*
Circle (Diameter)	Allows you to add a circle to the image.	
Ellipse	Allows you to add a ellipse to the image.	
Contour (Spline)	Allows you to add a contour (spline) to the image.	
Profile	Adds an intensity profile along a drawn in arrow region.	

Menu item	Description	Short cut
Rectangle Profile	Adds an intensity profile within the drawn in rectangle region.	
Scaled Profile	Adds an intensity profile with an scaling bar to the image.	
Grid	**Activated:** Adds a grid to the image.	
Frequent Annotations	Adds frequently used annotations to the image, e.g. Relative Time, Channel Name.	
Distance	Adds distance annotations to the image, e.g. Length, Curve length.	
Multiple Distances	Allows you to add annotations for measuring multiple distances to the image.	
Region	Allows you to add region annotations to the image, e.g. Contour, Rectangle.	
Circle	Allows you to add circle annotations to the image.	
Angle	Allows you to add angle annotations to the image.	
Points	Allows you to add points of interest (POI) to the image such as events or markers.	
Format	Allows to open the dialog for formatting graphical elements. Also you will find options for resetting customized graphical elements.	
Burn-in Annotations...	Creates a new image with all annotations burned-in to the image.	
Show Bounding Box	Shows bounding boxes around graphics/annotations.	
Hide Bounding Box	Hides bounding boxes around graphics/annotations.	
Bring to Front	Brings selected graphic/annotation to the front of the image.	
Send to Back	Sends selected graphic/annotation to the back of the image.	
Bring Forward	Brings selected graphic/annotation one layer forwards.	
Send Backwards	Sends selected graphic/annotation one layer backwards.	

13.1.6 Macro Menu

Info

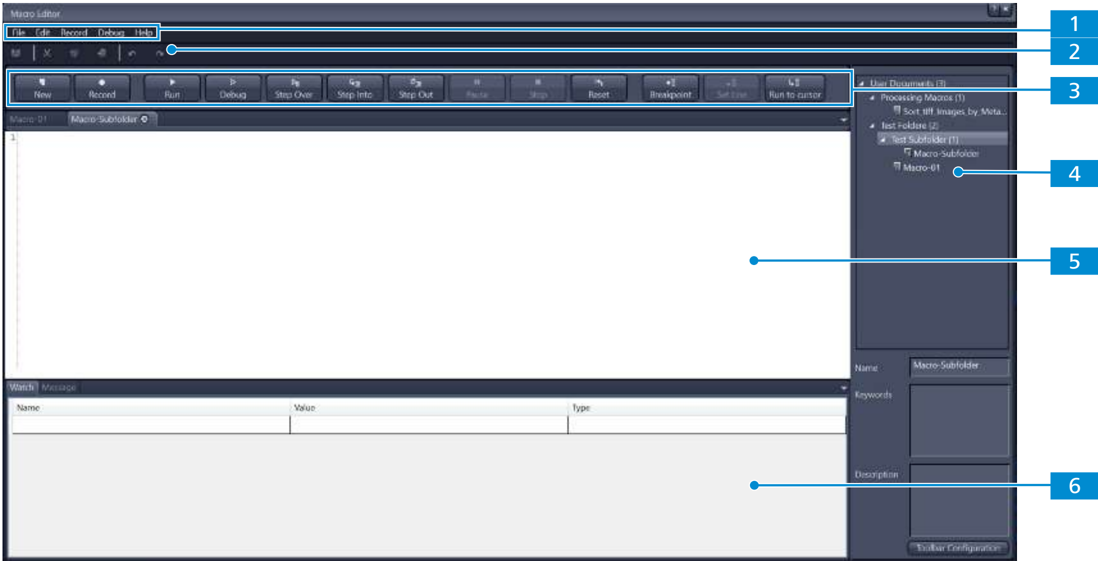
This menu is available only if you have licensed the **Macro Environment** module.

- Activate the Macro Environment controls in the **View** menu by klicking on **Show Macro Environment**.

Menu item	Description
Record a New macro	Starts recording a new macro.
Stop Recording	Stops recording the macro.
Macro-Editor…	Opens the *Macro Editor Dialog [▸ 622]*.

13.1.6.1 Macro Editor Dialog

The macro editor represents the IDE (Integrated Developer Environment) to edit, execute, debug and manage macros. It is started via the menu **Macro > Macro Editor** or from the **Macro** tool in the **Right Tool Area**.



1 **Menu bar**
For a detailed description of the menus, please read *Macro Editor Menus [▸ 623]*.

2 **Tool bar**
With the icons you can quickly access the most important functions, like saving or editing macros.

3 **Button bar**
Here you find the buttons to record and control macros. For more information, see *Button Bar [▸ 625]*.

4 **Macro list**
Displays all macros and folders. The macros can be moved into a different folder via drag and drop. Macros and folders can be edited via right-click context menu, see also *Macro List Context Menu [▸ 625]*.

5 **Code Window**
The central area of the Macro Editor shows the program code of the selected macro. Edit and write your macros in here. You can either use the **Record** button or type in the program code directly. Also a multi-document view is available, meaning that you can open several code windows at once.

6 **Watch/ Message Window**
Here you can observe variables of the macro program code. Enter the variable directly in the column **Name**. You can also mark the variable in the macro and add it using **Add Watch** of the right mouse key context menu.
The **Message** window displays messages when using the print command in a macro.

13.1.6.1.1 Macro Editor Menus

File Menu

Menu item	Description	Short cut
New Macro	Opens the **New Macro** in the Macro programming area.	
Import	Opens a file browser to select and import a macro.	
Save	Saves the selected macro.	*Ctrl+S*
Save As…	Saves the macro under a new name.	
Rename…	Opens the **Rename** dialog window. Enter a new name for the macro.	
Delete	Deletes the selected macro.	
Close	Closes the selected macro.	

Edit Menu

Menu item	Description	Short cut
Cut	Cuts the selected line out of the macro.	*Ctrl+X Shift +Del*
Copy	Copies the selected line in the macro.	*Ctrl+C Ctrl+Ins*
Paste	Inserts the copied line into the macro.	*Ctrl+V Shift+Ins*
Find	Finds the entered text.	*Ctrl+F*
Replace	Replaces the detected text with the new text..	*Ctrl+H*
Undo	Undoes the last action.	*Ctrl+Z*
Redo	Redoes the last action.	*Ctrl+Y*

Record Menu

Menu item	Description
Record	Starts recording.
Stop Recording	Stops the recording of the active macro.

Debug Menu

Menu item	Description	Short cut
Start Debugging	Starts the debugger and executes the macro up to a breakpoint or error.	*F5*
Start Without Debug-ging	Executes the macro up to a breakpoint or error without debugging.	*Ctrl+F5*
Pause	Pauses debugging.	*Shift+F5*
***Continue (DEBUG)**	Continues debugging.	*Shift+F5*
Stop	Stops the running macro at the active command.	*Shift+F5*
Step Into	Starts the debugger stepwise, command by command, without stepping into function blocks.	*F11*
Step Over	Starts the debugger stepwise, command by command, and steps into function blocks.	*F10*
Step Out	Starts the debugger stepwise, command by command, and steps out of function blocks.	*Shift +F11*
Toggle Breakpoint	Sets/removes a breakpoint in the active line to stop/ continue the macro in debug mode.	*F9*
Set Line To Execute	Sets the pointer in the next active command line.	*F8*
Reset	Resets all variables of the Python interpreter.	

Help Menu

Menu item	Description	Short cut
Contents...	Opens the **Online Help** dialog.	*Ctrl+F1*
Macro Object Model…	Opens the **Macro Object Model Online Help** dialog. This documentation includes descriptions of all objects available for the macro editor.	
Forum…	Opens the OAD forum in your web browser. Internet access required.	
GitHub	Opens the ZEISS GitHub page for OAD in your web browser. Internet access required.	

13.1.6.1.2 Button Bar

On this bar you find the buttons to record and control macros.

Parameter	Description
New	Creates a new empty macro.
Record	Starts macro recording.
Run	Executes the active macro completely.
Debug	Starts the debugger and executes the macro up to a breakpoint or error.
Step Over	Starts the debugger stepwise, command by command, without stepping into function blocks.
Step Into	Starts the debugger stepwise, command by command, and steps into function blocks.
Step Out	Starts the debugger stepwise, command by command, and steps out of function blocks.
Pause	Pauses macro recording.
Stop	Stops the running macro at the active command.
Reset	Resets all variables of the Python interpreter.
Breakpoint	Sets/removes a breakpoint in the active line, to stop/continue the macro in debug mode, in the active line.
Set Line	Sets the pointer in the next active command line.
Run to cursor	Sets the pointer to the current cursor position.

13.1.6.1.3 Macro List Context Menu

Folder menu

This context menu is only displayed if you right-click a folder in the Macro list.

Parameter	Description
Add Macro	Adds a new macro to the folder.
Add Folder	Adds a new subfolder.
Rename	Allows renaming the folder.
Delete	Deletes the folder.
Replace with Content	Deletes the folder and moves all macros in the folder into the next higher folder.
Expand All	Only available for folder on the top level. Expands all (sub-)folders.
Collapse All	Only available for folder on the top level. Collapses all (sub-)folders.

Macro menu

This context menu is only displayed if you right-click a macro in the Macro list.

Parameter	Description
New	Creates a new macro.
Save	Saves the current changes.
Save as	Saves the macro with a new name.
Rename	Allows renaming the macro.
Delete	Deletes the macro.

13.1.7 Tools Menu

Menu item	Description
Axio Scan Calibration…	Only available for Axio Scan systems. Opens the **Axio Scan Calibration** wizard. By the wizard you can calibrate the following functions: ▪ mapping of the preview camera to the scan camera, ▪ parfocality of the objectives, ▪ parcentricity of the objectives. ▪ shading correction of the optical system (including fluorescence) and color calibration. It is not necessary to execute all steps, e.g. the shading correction for fluorescence and the color calibration can be skipped.
Diagnostics	Opens the **Diagnostics** dialog. There you receive detailed reports on your entire system state. *Ctrl+Shift+D*
Kitchen Timer…	Opens the **Kitchen Timer** dialog. There you can set a time period after which an alert is played.
Dosimeter…	Opens the **Dosimeter** dialog. There you can set multiple time points at which an alert is played.
Dye Editor…	Opens the *Dye Editor Dialog [▸ 55]*.
Extension Manager…	Opens the **Extension Manager** dialog.
Modules Manager…	Opens the *Modules Manager [▸ 627]* dialog.
Users and Groups...	Opens the *Users and Group Management [▸ 627]* dialog.
Settings Editor...	Opens the **Settings Editor** dialog. Select from existing hardware settings or adopt the settings from the hardware being used into the software. You can also transfer settings from the software to the hardware that you are using.
System Maintenance and Calibration...	Opens the **System Maintenance and Calibration** dialog. Helps to keep your system in perfect working condition.

Menu item	Description
Calibration Manager…	Opens the **Calibration Manager** dialog.
Customize Application…	Opens the *Customize Application [▸ 627]* dialog.
Scaling…	Opens the *Scaling [▸ 629]* dialog.
Sample Carrier/Holder Templates…	Opens the **Sample Carrier Templates** dialog.
Options…	Opens the *Options Dialog [▸ 631]*.

13.1.7.1 Modules Manager Dialog

Here you can activate or deactivate the modules for which you currently own a license. Note that all the changes made here are implemented immediately.

Parameter	Description
Available Products	Here you can see the products available for your license. Click on the relevant button to select the product.
Included Modules	In this list you can activate/deactivate the modules that are included with your product. Activate the checkbox to activate the corresponding module.
Optional Modules	In this list you can activate/deactivate the modules that you have licensed as an option for your product.
Optional Hardware	In this list you see the hardware that you have configured.
Select All	Activates all available modules.
Unselect All	Deactivates all available modules.
Save Information...	Saves the current selection of modules within a **.txt** file.

13.1.7.2 User and Group Management Dialog

Here you can create new users and groups and manage their access rights.
Activate the user and group management by activating the checkbox **Enable User Management**. For more details read also the chapter Managing Users and Groups.

See also

- Creating a new user [▸ 37]
- Adding users to a group [▸ 38]
- Managing access rights for user groups [▸ 40]

13.1.7.3 Customize Application Dialog

Here you can customize the application layout, e.g. adopt the toolbar or shortcuts.
To learn more about how to customize the application, read the chapter *Customizing Toolbar [▸ 237]*.

Toolbar Tab

Here you can add menu items to the **Toolbar** as buttons for a quick access.

Parameter	Description
Available Toolbar items	In this list you see all menu items that you can add to the **Toolbar**.
Add	Adds a selected item to the tool bar. It then appears in the **Selected Toolbar Items** list.
Selected Toolbar Items	In this list you see all the added menu items. Select the items here in order to sort them.
Delete	Deletes a selected item from the **Selected Toolbar Items** list.
Up	Moves a selected item one position up in the **Selected Toolbar Items** list.
Down	Moves a selected item one position down in the **Selected Toolbar Items** list.
Separator	Inserts a vertical separator into the **Toolbar** after the currently selected item of the **Selected Toolbar Items** list.
Close	Closes the **Customize Application** dialog and saves the adjustments.

Shortcuts Tab

Parameter	Description
Available Commands	In this list you see all commands from the **Menubar** and edited Macros. Klick on the arrow on the left of the entry to show available commands.
Shortcut for the selected item:	If you have selected a command from the **Available Commands** list, the related shortcut is displayed here. If the field is empty, no shortcut yet exists for the selected command.
Remove	Deletes a shortcut of the selected command. Default shortcuts, e.g. *Strg+S* can not be removed.
Type a shortcut	Here you can type in a shortcut by clicking on the desired keys of your keyboard. If a shortcut is already used for another command, it is displayed in the **Shortcut is used by:** display field.
Add	Adds a shortcut to a chosen command.
Shortcut is used by:	If you typed in a shortcut which is already used, the related command is displayed here.
All commands with shortcuts:	In this list you see all the shortcuts and their related commands.
Close	Closes the **Customize Application** dialog and saves the adjustments.

Soft Keys Tab

Parameter	Description
Available Items list	In this list you see all items from the **Menubar**, Hardware Settings and edited Macros. Klick on the arrow on the left of the entry to show available items.
Soft Keys	The items from the **Available Items** list can be assigned to the buttons **Function0**-**Function9** via drag & drop.
Reset All	Resets all the adjustments.
Close	Closes the **Customize Application** dialog and saves the adjustments.

13.1.7.4 Scaling Dialog

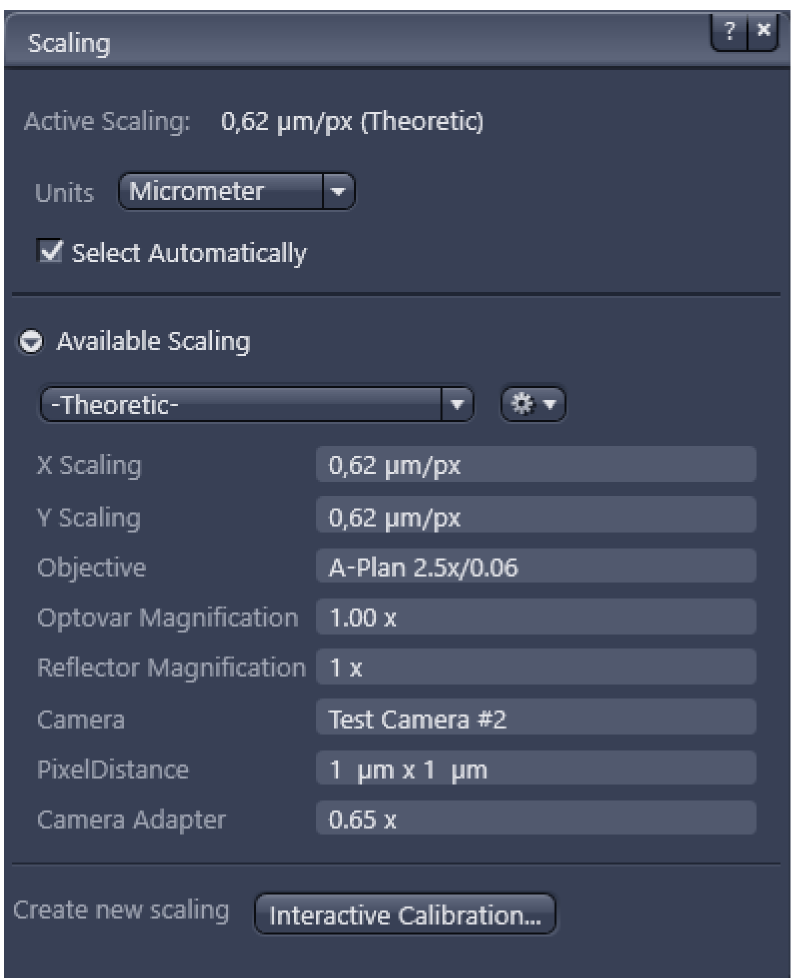


Fig. 48: Scaling Dialog

Here you can specify how your images are scaled.

Parameter	Description
Active Scaling:	Shows the scaling that is set currently.
Units	Select the desired unit for the current scaling here.

Parameter	Description
Select Automatically	**Activated:** Calculates the scaling automatically from the microscope and camera configuration.
Scaling	Select scalings which are stored on your system e.g. Pixel, Theoretic. The scaling details will be displayed in the fields below the list. If a display field is empty, it will not be used in the calculation of the scaling.
Options	By clicking on the you can perform the following actions :
- Activate Scaling	Activates the selected scaling. The scaling will be applied to all images that are acquired from this time point onward.
- Assign Scaling to Image	Assigns the selected scaling to the current image.
- Import	Opens the **Import Scaling** dialog window. Here you can select a scaling file (.czsc) that you want to import.
- Export	Opens the **Export Scaling** dialog window to export the selected scaling. Select the folder in which you want the exported scaling file to be saved and specify a file name (.czsc).
- Delete	Deletes the selected scaling.
Interactive Calibration...	Opens the **Open file for interactive scaling** dialog, if there is no image yet selected. Starts the *Scaling Wizard [▸ 630]* in the **Center Screen Area** for the currently selected image.

13.1.7.4.1 Scaling Wizard

Here you can create a new scaling. To do this, draw a reference line with a predefined length in the current image. An image of a calibration slide is best suited for this purpose.

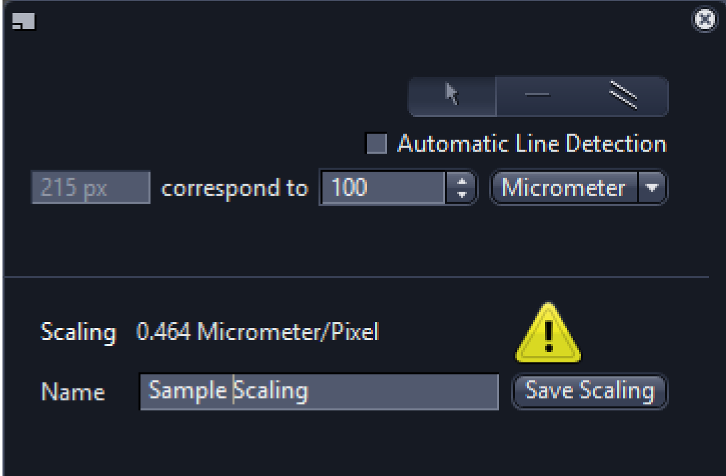


Fig. 49: Scaling Wizard

Parameter	Description
Tool Bar	Here you can draw in two types of reference line. Therefor klick on one of the following buttons.

Parameter	Description
- Select	If selected, the courser is in selection mode. You can move the dialog window or select a reference line to edit it.
- Draw Reference Line	This tool is selected by default. With that tool you draw in a line along a distance with a known length (e.g. using an object micrometer or calibration slide). Then you can adjust the length and units in the input field and dropdown list.
- Draw Parallel Reference Line	With that tool you draw in two parallel lines along a distance with a known length. The two parallel lines correct errors in the parallel axis resulting from the drawing of the lines. A third, corrected line is drawn in automatically from which the scaling is determined.
Automatic Line Detection	**Activated:** Automatically detects individual lines of the scale bar in the image close to the interactively defined distance. Using this method the centers of the lines are determined exactly, increasing the precision of the scaling.
Length input field	Here you must enter the length of the line you have drawn in.
Unit selection	Here you must select the corresponding unit of your drawn in line.
Scaling	Shows the calculated pixel scaling according to the drawn in line.
Name	Here you can enter the name for the scaling that will be created.
Save Scaling	Saves the scaling that has been created under the specified name. The scaling can be selected in the **Scaling** dialog under **Available Scalings**.

13.1.7.5 Options Dialog

Here you can configure the settings for general software options.

13.1.7.5.1 General Tab

Parameter	Description
Select Automatically	**Activated:** Automatically selects the user language of the operating system as the user language for the software.
Fixed Language	Select the language from the dropdown list in which the software will be run next time it is started.

13.1.7.5.2 Startup/Shutdown Tab

Parameter	Description
Show Splash Screen	**Activated:** Displays the splash screen when the software starts.
Show Application Selection	**Activated:** Shows the application selection dialog when the software starts.

Parameter	Description
Reload Last Used Documents	**Activated:** Reloads all image documents, that were open when you last exited the system when the software starts.
Experiment	Selects the desired bahaviour on system start up with regard to the experiment management on the **Acquisiton** tab.
- **Load default experiment**	Loads an experiment with the set default values.
- **Load empty experiment**	Loads an empty experiment.
- **Load last used experiment**	Loads the last used experiment.
Request Stage/Focus Calibration on Startup	**Activated:** Shows a message which asks you to perform stage/focus calibration.

13.1.7.5.3 Naming Tab

Here you can specify how images are (automatically) named and indexed. Changes will be stored after the session is ended.

Parameter	Description
Category	Select the category of the file you want to be named automatically, e.g. an Image or an Experiment.
Prefix	Here you can enter a prefix for the file name, e.g. IMG.
Digits	Select how many digits you want the counter to have.
Format	Here you can specify what information you want to include to the file name. From the **Format-IDs** list below you can add the desired attribute to the name. Therefore simply double click on the desired format in the list.
Initial Counter Value	Here you can enter the desired first value of the counter.
Suffix	Here you can enter a prefix for the file name.
Preview	Displays the preview of the naming format that will be allocated next for the selected category.
Save/Restore Counter Value	**Activated:** Saves the counter values for the individual categories. If the software is restarted, the values are restored.
Format IDs	Shows all the attributes which can be used for the **Format** field.

13.1.7.5.4 Saving Tab

Parameter	Description
Auto Save after Snap	**Activated:** Automatically saves images that are acquired on the **Locate** tab using the **Snap** button.

Parameter	Description
	Deactivated: Saves images that are acquired on the **Locate** tab using the **Snap** button temporarily in the Auto Save Path\Temp. The image will be marked with an asterisk and will be deleted if it is closed without saving.
Don't Open a Document Window	Only active if the **Auto Save after Snap** checkbox is activated. **Activated:** Closes the automatically saved images immediately after acquisition.
Auto Save Path	Specifies the folder where the images are saved automatically.
Show "Discard All" Button in Dialog to Save Modified Documents	If activated, the **Discard All** button is displayed in the **Save Documents** dialog. **NOTICE! If you click on this button all un-saved images will be deleted.**

See also

Auto Save Tool [766]

13.1.7.5.5 Documents Tab

Default Settings for New Images section

Parameter	Description
Show Rulers	**Activated:** Displays rulers at the top and left-hand edge of the image – the units used are according to the scaling settings.
Auto Fit	**Activated:** Automatically adjusts the zoom factor of the image so that the entire image is visible and the view area is filled.
Use Interpolation for Image Display	**Activated:** Displays pixels in interpolated form.
Set Logarithmic Scale in Histogram	**Activated:** On the **Display tab** the frequency distribution (y-axis) of the histogram is plotted using a logarithmic scale.
Show Viewport Scale-bar in 2D View	**Activated:** Shows a scale bar within a small window in 2D view.
Show Viewport Scale-bar in Live Window	**Activated:** Shows a scale bar within a small window in the Live window.
Show Navigator in 2D View	**Activated:** Shows the Navigator window in the image area.
Use Pan Mode in 2D View for Tile Images	**Activated:** Pan mode will autoatically activated for tiled images opened in 2D view.

Display section

Parameter	Description
Enable Tree View	Additionally shows the **Tree** view in the **Center Screen** area.

Parameter	Description
Show Time series/ movie images without Bounding X/Y Area	**Activated:** Time series or movie images acquired when the stage co-ordinate is adjusted are shown without the bounding X/Y area (black boarder).
Show Rotation Slider in Dimensions tab	Shows the Rotation Slider in the **Dimensions Tab**.

Image Rendering

Parameter	Description
Use Advanced Renderer for Image Acquisition (Note that in a few instances display artifacts can occur)	Usually the advanced renderer is deactivated during acquisition. This check box activates the renderer during acquisition, which can cause an appearance of display artifacts.

3D View section

Parameter	Description
Run Performance Assessment	Runs a test routine which evaluates the performance of the graphic card installed on the workstation. The result is an adjustment of the precision and accuracy parameters to allow fluid interaction with the rendered volume in 3D view.
Graphics Hardware Class	Selects the performance class of your graphics hardware. A higher performance class allows you to see your data in more detail but may lead to crasheson unsuitable hardware. The following classes are available: ▪ **Very Low** ▪ **Low** ▪ **Normal** ▪ **High** ▪ **Very High**
Interactive Precision	Controls the interactive precision in %.
Interactive Accuracy	Controls the interactive accuracy in %.
Show Logo	**Activated**: Logo is displayed in the lower right corner of the 3D view.
Export Logo	**Activated**: Exports the logo in render series and snapshots.

13.1.7.5.6 Acquisition Tab

General section

Parameter	Description
Automatically Add Scalebar Annotation at Snap	**Activated:** Automatically adds a scale bar to the image, if it was acquired via the **Snap** button.
Show a Request to Move Manual or Coded Hardware Components	**Activated:** Shows a dialog which asks you to move manual components. You have to confirm the dialog and move the component by hand.
Show a Confirmation Dialog for Channel/Track Deletion	**Activated:** Shows a dialog which asks you to confirm to delete a channel or a track.
Lock Device Controls in Right Tool Area During Running Experiments	**Activated:** Prevents controls in the right tool area from be undocked during an experiment.

Camera/Live section

Parameter	Description
Stop Live after Snap	**Activated:** Automatically closes the **Live** mode after an acquisition via the **Snap** button.
Stage/Focus Control in Live/Continuous View	Enables to navigate the stage and focus in **Live** and **Continuous** view. Configure the travel speed of the focus by adjusting the values in the corresponding fields from **Very Slow** = 0,005 to **Very Fast** = 50,0. Reset your adjustments by clicking on the **Default** button.
Show Camera Expert Options	**Activated:** Shows advanced (expert) camera options on **Locate** tab within the **Camera** tool.
Use Centered Camera ROI only	**Activated:** Positions a camera ROI at the center of the camera chip regardless of its size. Centered Camera ROI = center of camera detector

Acquisition Tab section

Parameter	Description
Acquisition Tab without channel support	**Activated:** Enables the use and set-up of experiments without any channel support in the **Acquisition** tab.
Prevent Execution of After Channel Setting while Live Mode is Active	**Activated:** Prevents execution of after channel setting automatism while **Live** mode is active.

Parameter	Description
Automatically start Live Mode when Exposure Measure-ment was Started	**Activated:** Starts the**Live** mode when the **Set Exposure** button has been pressed such that the live image begins immediately after the **Set Exposure** measurement is complete. **Deactivated:** Takes a **Snap** subsequent to **Set Exposure**.
Switch to next En-abled Acquisition Block in Experi-ment Designer	**Activated:** Automatically switches to the next enabled acquisition block in the Experiment Designer during a running experiment.
Enable Imaging Setup	Shows the **Imaging Setup** tool on **Acquisition** tab.
Enabled Advanced Imaging Setup	Only active, if the **Enable Imaging Setup** checkbox is activated. **Activated:** Shows the **Standard/Advanced** option on the **Acquisi-tion** tab on top of the **Imaging Setup** tool. As the advanced options should be touched by experts only, this option is deactivated per de-fault.

Z-Stack section

Parameter	Description
Adjust Auto-Z-Stack Focus Match on First Slice	Determines the degree of match between the image focus of the first image and that determined as the true focus (center plane of the re-sulting Z-stack).
Adjust Auto-Z-Stack Focus Match on Last Slice	Determines the degree of match between the image focus of the last image and that determined as the true focus (center plane of the re-sulting Z-stack).
Delay Time After Focus Move	Specifies a delay time after each focus movement during Z-Stack ex-periments in ms.

Tiles & Positions section

Parameter	Description
Automatically Start Live Mode in the Advanced Setup View	**Activated:** Automatically starts the **Live** mode in the **Center Screen Area** if you klick in the **Acquisition** tab in the **Tiles** tool on the **Ad-vanced Setup** button. Uncheck this option to prevent unnecessary specimen bleaching. The default is not activated.
Automatic Snap by Clicking the Live Navigator Buttons	**Activated:** Acquires an image if you click on one of the frame's blue arrow icons. The **Live Navigator** tool moves one frame width in the relevant direction. You can create tile images of your sample easily in this way.
Enable Stage Movement with Live Navigator	In the Live navigator tool the current stage position including the live image is shown as a frame outlined in blue. To move the frame, dou-ble-click on the position to which you want to move it. Alternatively, place the mouse cursor over the blue frame, press and hold the left mouse button and drag the live navigator to the desired location. **Activated:** Allows you to move the **Live Navigator** tool by dragging it to a new location.

Parameter	Description
Show Stage and Focus Backlash Correction Setting in the Options	**Activated:** In the Tiles option, the setting to switch the backlash correction on or off is shown. Per default it is hidden.
Delimiter for CSV Export/Import	Specifies the delimiter for a CSV export or import. Select **Comma** (default), **Semicolon** or **Tab**.
Ask Whether Support Points/Positions Should be Overwritten	When the support points and/or positions are determined by a software autofocus run the existing points can be overwritten with the new **Z** values. **Activated:** Shows a message box asking if the points should be overwritten if there is a autofocus **Z** value.
Focus Surface Outlier Determination	Ignores support points that are significantly outside the interpolated focus surface. You have the following setting options available:
- Maximum Interpolation Degree for Outlier Detection	This value can be 0 or 1. If 1 then a linear fit is used to detect the outlier support points. This is the default. If 0 a simple average value is used to detect outliers.
- Threshold in Terms of the Standard Deviation (Sigma)	This parameter defines a threshold value to determine which of the support points are outliers from the fitting process. This is defined by the standard deviation (sigma value) set in the spin box. Support points not meeting this criteria are subsequently ignored when the focus surface is determined.
Delay Time After Stage Movements	Defines a delay period which is used for all stage movements in a tiles and position experiment or movements controlled in the advanced tile setup. The delay helps prevent movement in samples where, for example, a large volume of liquid is present in the sample holder. It can be used with the stage speed and acceleration options to optimise experiments with this type of sample.
Binning Compensation of Exposure Time in Preview Scans	Defines the power to which the binning ratio is modified to automatically determine the exposure time value used for a preview scan were the binning setting between the experiment and preview scan differs. The default value is 2.0 i.e. quadratic. Thus, for example the exposure time would be reduced by a factor of four if the experiment binning is 1x1 and the preview scan binning is 2x2. The value can be varied between 1.0 and 2.0 in steps of 0.1.
Live Image in Sample Carrier Calibration Wizard (relevant only for systems with camera)	
- Use Imaging Device from Selected Channel with "Acquisition" Settings	**Activated:** Default setting for the live image that allows navigation and focus interaction during the carrier calibration wizard.

Parameter	Description
- Use Active Camera with "Locate" Settings	This option is only relevant for systems with a wide field (camera based) detector. **Activated:** Allows you to alternatively apply locate camera settings for use in the carrier calibration wizard (live image). By default the experiment settings for the currently selected channel/track will be used.

Panorama section

Parameter	Description
Automatically Start Live Mode in the Panorama View	**Activated:** Specifies that the live mode will start running automatically when you begin a panorama experiment.
Automatically Move Stage/Live after an Acquisition	**Activated:** Automatically moves the stage half a camera frame diagonally after acquisition of a snap image. Thus, the snap image can be inspected.
Enable Transparency Effect on Selected Tile Image	**Activated:** Displays the selected tile image with a transparency effect that enables you to see it in relation to the tiles underneath (lower layer = earlier acquisition) and those above (upper layer = more recent acquisition) at the same time. You can also adjust the degree overlap of the panorama grid. The default value is 20%, changes require a re-start to become affective. Note that this and the transparency effect parameters are only relevant for manual stages.
Grid Overlap	Specifies the degree of overlap for the panorama grid in %. A software restart is required.
Show Live Panorama Acquisition Options	**Activated:** Note that the option should only be activated, if you have issues with the camera / live image during acquisition. It is only available for troubleshooting and normally not needed. **Not activated:** Default. The software tries to use the determined value for the current camera automatically. If you are unsure, if your camera supports the functionality, we recommend leaving the default.

Focus Strategy section

Parameter	Description
Show a Dialog to Prepare the Definite Focus Initialization	**Activated:** Reminds you to make appropriate adjustments to the focus prior for initialization at the start of experiments using Definite Focus.
Enable Definite Focus Stabilization on a Suitable Fallback Position	**Activated:**Specifies whether the last successfully determined z position is used if the primary focus action (Definite Focus or Autofocus) fails.
Show Definite Focus Setting "Resolution and Speed"	**Activated:** An additional section is displayed in the definite focus strategy. This allows selection of three definite focus modes for greater speed or accuracy of the stabilization.

Experiment Feedback section

Parameter	Description
Time until a deadlock of the synchronized script is assumed	Sets the time until a deadlock of the synchronized script is assumed. Here you can adjust the time until ZEN assumes that a deadlock has occurred after a synchronized script has been executed. If this value is exceeded the function is aborted.

Mixed Mode Options section

Parameter	Description
Activate the online disparity warping	Specifies whether the disparity map is used to warp the LSM and Airyscan frames.
Path to disparity map	Displays the path where the disparity map is saved.
Interpolation Mode	Sets the interpolation mode.
– Nearest Neighbor	The output pixel is given by the gray value of the input pixel that is closest to it.
– Linear	The output pixel is given by the gray value resulting from the linear interpolation of the input pixels closest to it.
– Cubic	The output pixel is given by the gray value resulting from a cubic polynomial function interpolation of the input pixels closest to it.
Disparity Map Calibration Integrates the Radial Distortion	**Activated:** Integrates the radial distortion for the calculation of the disparity map. **Note:** This option should not be deactivated because it will have a negative effect on the quality of the results.

LSM section

Parameter	Description
Online Scanner Correction	**Activated:** Enables the online scanner correction. It ensures an optimal image quality at scan speeds > 13.
Airyscan Processing Baseline Shift	**Activated:** Adds an offset of 10.000 to the processed Airyscan images. This allows to display details in the processed Airyscan image that have negative intensity values and are therefore normally cut from the histogram.
Bidi Auto Correction	Automatically corrects line shifts for bidirectional scanning for Multiplex Imaging modes
Set lasers OFF when unused for 30 minutes (requires ZEN restart)	When activated any laser which is not activated for an experiment will automatically be set to OFF mode after 30 minutes. This does not apply to multiphoton lasers.
Activate extended zoom range	Activates the extended zoom range.

Parameter	Description
Enable Z-Piezo	**Activated:** Uses the Z-Piezo drive (if available) for the acquisition of Z-Stacks. Stacks with a range that is larger than the specified working range of the piezo drive are automatically carried out using the microscopes focus drive.
Z-Piezo Range	Select the range of the Z-Piezo drive. Note that the precision in the high range is lower compared to the default range. **Note:** This function is not available with LSM 980. With LSM 980 the range of the Z Piezo is always 500 µm with high precision.

13.1.7.5.7 User Tab

Here, you can enter user and company information. These are written into the image metadata during acquisition.

Parameter	Description
User Information	Type in contact information of the software user here.
Company Information	Type in contact information of the company/institute/facility here.
Logo	Upload a company logo to the company profile here. Therefore click on the ... button.

13.1.7.5.8 Data Tables Tab

Data Table Import Options section

Parameter	Description
Start Import in Row No.	Defines the starting row of the data table into which the data will be imported.
Automatic CSV format detection	**Activated:** Automatically tries to detect the format of the data table when importing the table to the software.
Use first imported row as column caption	**Activated:** Uses the first imported row as the caption for the column.
Use second imported row as column unit	**Activated:** Uses the second imported row as the unit for the column.
Use column, decimal and list separator from windows regions settings	**Activated:** Uses the settings which are configured in the Windows regions settings when importing a table to the software.
Column Separators	Active only if you have deactivated the checkboxes **Automatic CSV format detection** and **Use column, decimal and list separator from windows regions settings**. Configures the import options according to the format of your data table you want to import, e.g. specify the type of column or decimal separator.

Parameter	Description
Decimal Separator	Active only if you have deactivated the checkboxes **Automatic CSV format detection** and **Use column, decimal and list separator from windows regions settings**. Select here, which decimal separator should be used.
Thousands Separator	Active only if you have deactivated the checkboxes **Automatic CSV format detection** and **Use column, decimal and list separator from windows regions settings**. Select here, which thousands separator should be used.

Data Table section

Parameter	Description
Number of Decimal Places	Set the maximum number of decimal places for the numbers imported into the data table here.

13.1.7.5.9 Macro Editor Tab

Macro Configuration section

Parameter	Function
Show Inherited Members in Pop-up	**Activated:** Shows the inherited members of the ZEN class in a pop-up window.
Show line Numbers	**Activated:** Shows the line numbers.
Disable fullscreen mode while debugging	**Activated:** Disables the fullscreen mode during debugging.

TCP Macro section

Parameter	Description
TCP Macro Section	**Activated:** Enables to enter the **TCP Port Number**.
Allow IPv4 Nat Traversal	Attention! For experts only. Do only activate this option if you know what you are doing.

Macro Recorder

Parameter	Description
Overwrite Interactive Recording Flag	**Activated:** Overwrites the parameter for interactive execution of a function during recording with the macro recorder.

13.1.7.5.10 APEER Tab

Parameter	Description
API Key	Sets/displays your private API Key which is required for the use of the APEER functionalities within ZEN. For more information, see *Creating and entering an API key [▸ 245]*.
Choose Execution Mode	Selects if the execution should be done locally or on a remote server. Available options: ▪ **Use Local Docker Desktop** ▪ **Use Remote Docker Host**
Default Execution Location	Only visible if **Use Local Docker Desktop** is selected. Selects a default location where the APEER module is executed and the outputs are saved.

Parameter	Description
API Key	Sets/displays your private API Key which is required for the use of the APEER functionalities within ZEN. For more information, see *Creating and entering an API key [▸ 245]*.
Choose Execution Mode	Selects if the execution should be done locally or on a remote server. Available options: ▪ **Use Local Docker Desktop** ▪ **Use Remote Docker Host**
Default Execution Location	Only visible if **Use Local Docker Desktop** is selected. Selects a default location where the APEER module is executed and the outputs are saved.

| – Use Docker Desktop with WSL 2 | Only visible if **Use Local Docker Desktop** is selected.
Activated: Docker Desktop uses WSL 2 (Windows sybsustem for Linux). |

Remote Docker Options

Only visible if **Use Remote Docker Host** is selected.

Parameter	Description
Remote Docker Host API Address	Sets the address and port of the remote Docker host.
Check Connection	Checks if the remote Docker host can receive a request. A green checkmarks indicates a successful connection, otherwise a red **x** is displayed.
Choose Authentication Mode	Selects the mode how you have to authenticate yourself to the remote host.
– No Authentication	The remote host needs no authentication.
– Basic Authentication	Displays a field to enter the **Username** and **Password** required for the remote host. Also the checkbox to **Use TLS (https)**.

Parameter	Description
– Certificate File Authentication	Displays a field to browse for the **Certification File** and enter a **Password (optional)** required for the remote host.
– Windows Certificate Authentication	Displays a field to browse for a **Certificate from Windows Store** required for the remote host.
Local to Remote File System Mapping	Area to map a folder path from the local computer to the same path on the remote computer.
– Path List	Displays and maps the path of a folder on this machine to the path of the same folder on the remote Linux machine.
–	Deletes the currently selected path mapping.
–	Opens the *Edit Mapping Dialog [▸ 643]* to edit the currently selected path mapping.
–	Opens the *Edit Mapping Dialog [▸ 643]* to add a new path mapping.
Export Remote Settings	Opens a file browser to export the current settings as an xml file.
Import Remote Settings	Opens a file browser to select and import an xml file with settings for the remote setup.

Advanced Settings

Parameter	Description
Client Name	Sets a custom client name for this computer.
Ports that can be used for the Docker Containers	Defines the ports which can be used by the Docker containers.

See also

- APEER (on-site) [▸ 244]
- APEER Workflows (online) [▸ 252]

13.1.7.5.10.1 Edit Mapping Dialog

Parameter	Description
Local Computer Path	Selects the path to the folder on this local computer. Click on ... to open a file explorer and browse to the folder.
Remote Computer Path	Defines the path the same folder on the remote computer.
Check Mapping	Checks if the two paths point to the same folder and displays if the check is successful or not.
Ok	Saved this path mapping and closes the dialog.

Parameter	Description
Cancel	Closes the dialog without saving.

13.1.7.5.11 Direct Processing Tab

On this tab you have the options to set up the communication between PCs for Direct Processing. For more detailed information, see *Connecting the acquisition computer with the processing computer [▸ 344]* and for general information see the chapter for *Direct Processing [▸ 343]*.

Setup Acquisition PC

In this section you can set up the acquisition computer if you use a discovery proxy. For more information, see also *Connecting the computers with discovery proxy [▸ 345]*.

Parameter	Description
Find From Discovery Proxy	**Activated:** Displays the control to define a Discovery Proxy from which you can get a list of available PCs for processing.
IP Address (Discovery Proxy)	Only visible if **Find From Discovery Proxy** is activated. Sets the address of the Discovery Proxy from which you can get a list of available PCs for processing.

Setup Processing PC

In this section you can set up the processing computer.

Parameter	Description
Announcement to Discovery Proxy	**Activated:** Uses a Discovery Proxy server for the communication between the computers. For more information, see *Connecting the computers with discovery proxy [▸ 345]*.
IP Address (Discovery Proxy)	Only visible if **Announcement to Discovery Proxy** is activated. Sets the IP address for the Discovery Proxy.
IP Address (this PC)	Displays the IP address of your computer and allows you to change the **Port**.
PC Description	Here you can add specific information about the processing computer that will be visible on the acquisition computer.
Send Hardware Information (optional)	**Activated:** Displays the hardware information of this computer, which is also visible on the acquisition computer.
Send Processing Statistics (optional)	**Activated:** Displays Direct Processing statistics (processing speed), which is also visible on the acquisition computer.

Setup Discovery Proxy Server

In this section you can set up your own computer as a Discovery Proxy. For more information, see also *Setting up your PC as discovery proxy [▸ 346]*.

Parameter	Description
IP Address (this PC)	Displays the IP address of your computer.
Start/Stop	Uses your computer as Discovery Proxy. The button changes to **Stop** to stop acting as Discovery Proxy.
IP Address to clipboard	Only available if the **Start** button has been clicked. Copies the IP address of your computer to the clipboard.

13.1.7.5.12 ZEN Connect

Parameter	Description
Stage Size	
– Stage Size in mm	Positions the images initially in a better way in the correlative workspace. For example, for 130x100mm stage an image at stage position 65x50mm will be placed in the center of the correlative workspace. Note that the stage size will only be taken into account for better initial positioning if you have calibrated the stage upon startup or later, but before creating the ZEN Connect project.
Stage Move Confirmation	
– Deactivate Confirmation	**Activated:** No confirmation dialog is shown when the stage is moved with double click in the Correlative Workspace. **Deactivated:** A confirmation dialog is shown each time the stage is moved with double click in the Correlative Workspace.
3D Rotation	
– Show Outlines of Rotated Cube	**Activated:** Displays a yellow outline of a rotated image in the Correlative Workspace. This outline is only visible in alignment mode, if **3D Alignment** is selected and **Apply 3D Rotation** is activated.
2D Image Sets	
– Always display single plane data sets	**Activated:** Always displays single plane data sets in the correlative workspace, even if the z-value set by the **Global-Z** is out of range for the 2D image(s).

13.1.7.5.13 ImageJ Tab

This tab is only shown if the ImageJ extension is activated.

Parameter	Description
ImageJ Folder	Shows you where the software is installed.
ImageJ executable	Select your preferred .exe file in the drop down list.
Shift Pixels to 16 bit	**Activated:** Shifts the grey value of a 10-bit or 12-bit image to 16-bit. **Deactivated:** No shift takes place.

Parameter	Description
Assume macro has input	
– False	The software does not expect macro input.
– True	The software expects macro input.
Default preferred conversion	You can specify the default for the preferred conversion.
– Automatic	Uses the automatic conversion setting.
– Always	Image is always converted.
– Keep	Image is not converted.
Default preferred file format	You can select the default setting for the preferred file format. Available options in the drop down list: ▪ Automatic ▪ czi ▪ Ome Tiff ▪ Tiff ▪ Tiff With Display Mapping

13.1.7.5.14 Storage Settings Tab

Here you can configure the settings for the ZEN Data Storage.

Parameter	Description
Document Server Url	Sets the Url of the ZEN Data Storage server.
Reset to Defaults	Resets all settings for the ZEN Data Storage to their defaults.
Server Setup	Sets up the ZEN Data Storage server.

See also

Setting up the ZEN Data Storage [▸ 591]

13.1.8 Window Menu

Menu item	Description	Shortcut
Full Screen	Sets the **Full Screen** mode to maximize the image view area size.	*F11*
Next Window	Displays the next open image in the **Center Screen Area** (direction of movement is to the right).	*F6*
Previous Window	Displays the previous open image in the **Center Screen Area** (direction of movement is to the left).	*Ctrl+F6*

Menu item	Description	Shortcut
Close	Closes the currently selected image.	*Ctrl+F4*
Close All	Closes all the open images.	*Ctrl+Alt+W*

See also

Full Screen mode [908]

13.1.9 Help Menu

Menu item	Description	Shortcut
Contents…	Opens the online help contents page.	*Ctrl+F1*
Index…	Opens the online help index page.	*Ctrl+F2*
Readme	Opens the file path to the Readme PDF-file.	
Create Service Report...	Opens the dialog to create the service report. For more details, read chapter *Creating a Service Report [1049]*.	
About ZEN…	Shows notices of the producer.	

13.2 Main Tabs

13.2.1 Locate Tab

Depending on the system configuration and the licensed modules this tab can have a different appearance. In general you can use the locate tab for finding or "locating" interesting areas on your sample.

For mixed systems (e.g. LSM including a microscope camera) the section **System Mode** is available additionally.

In the **Eyepiece** mode (for confocal systems) this tab contains only functions for controlling the light path and viewing the sample via the eyepiece, see *Microscope Control Tool [▸ 666]*. In the **Camera** mode this tab contains more control elements and tools, see Tools on Locate tab

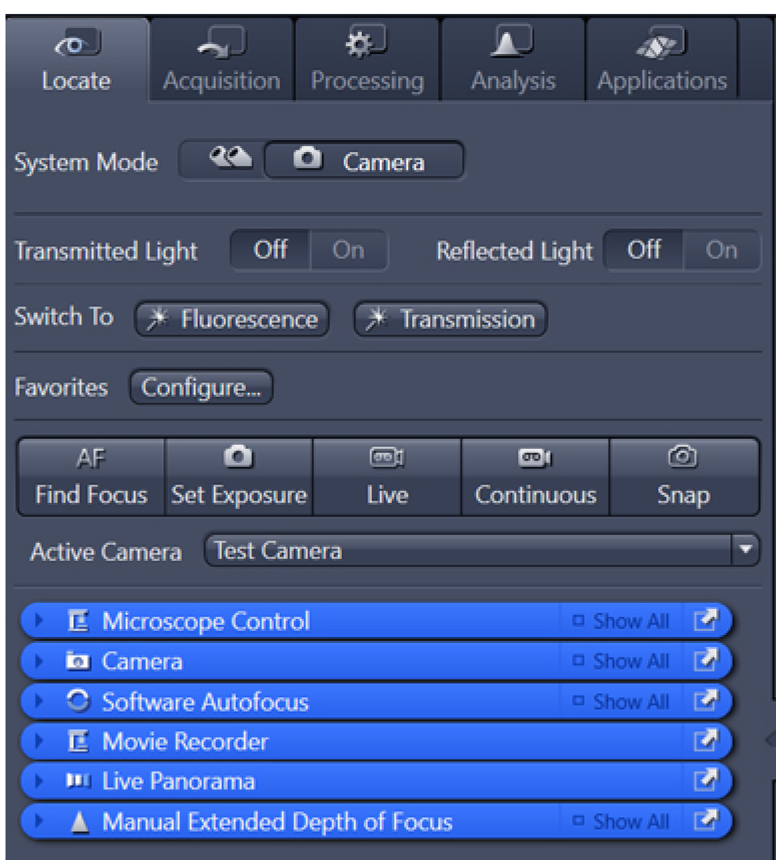


Fig. 50: Locate tab (Camera mode)

System Mode section

This section is only visible if a camera is installed with the system.

Here you can switch the system mode between **Eyepiece** and **Camera** mode. To switch between the 2 modes simply click on the corresponding button. In the table below you find a description of the two modes:

Mode	Description
Eyepiece mode	If you switch to Eyepiece mode the system adjusts the light path automatically to the eyepiece. The following list shows the changes in the ocular mode in detail: ▪ All **Action** buttons are hidden ▪ Only the **Microscope Control** tool is visible ▪ Within the **Microscope Control** tool only the light path leading to the eyepiece is displayed. ▪ All possible light paths to cameras are hidden.
Camera mode	▪ All Widefield relevant components are displayed as usual. ▪ The LSM Scan Head icon is not displayed in the light path.

Transmitted Light/Reflected Light section

Only visible if you have configured a motorized TL/RL shutter in MTB (MicroToolBox).

Parameter	Description
Off	Closes the shutter of the transmitted/reflected light source on a motorized microscope.
On	Opens the shutter of the transmitted/reflected light source on a motorized microscope.

Switch To section

These buttons configure the light path either for transmitted light observation or fluorescence observation. The observation is either via Ocular or Camera which is set in the **System Mode** section. The shutter for either observation path is not opened to avoid unwanted illumination of the sample. Shutter for either mode can be operated directly on the microscope hardware or using the controls in the Transmitted Light/Reflected Light section.

Mode	Description
Transmission	Click on the **Transmission** button to set the beam path in the microscope for observation of the sample using transmission illumination. This includes all motorized components of the microscope excluding the shutter in front of the transmitted light source. On a fully motorized microscope no further changes in HW are necessary apart from opening the light shutter and setting the illumination strength of the halogen lamp to achieve transmission observation of the sample. Note: When switching to **Acquisition** the light shutter is closed. When switching back to **Locate** the settings for Transmission observation will be restored.

Mode	Description
Fluorescence	Click on the **Fluorescence** button to open up the *Add Dye or Contrasting Method Dialog [▸ 809]*. Chose the dye you want to observe in fluorescence mode, then close the tool. The beam path is set for fluorescence observation including all motorized components of the microscope excluding the shutter in front of the reflected light source. On a fully motorized microscope no further changes in HW are necessary apart from opening the light shutter and setting the illumination strength of the reflected light source to achieve fluorescence observation of the sample. Note: When switching to **Acquisition** the light shutter is closed. When switching back to **Locate** the settings for Fluorescence observation will be restored.

Favorites section

Parameter	Description
Configure...	Here you can configure further buttons with your favorite hardware setting functions. Click on the button to open the Configuration dialog.

Action buttons section

With these buttons you control the microscope and the camera and acquire your images.

Fig. 51: Action buttons

Parameter	Function
AF **Find Focus**	Only visible if you have configured a motorized focus in the **MTB** (MicroToolBox). Starts an autofocus search using the current settings from the **Software Autofocus** tool.
Set Exposure	Starts an automatic exposure time measurement with the settings defined in the **Light Path** and **Camera** tool or (for LSM) the settings in the Imaging set up tool.
Live	Opens **Live View** and shows the live image from the active camera or the first channel of the first track when acquisition is performed with LSM.
Continuous	Starts a series of **Snaps** using the settings defined in the **Light Path** and **Camera** tool. In contrast to a live image, the exact same camera setting that has been set in the **Camera** tool is used. The result at the end of this mode is a single, acquired image that can be saved.
Snap button	Acquires a single image.

Parameter	Function
Stop	Only active if one of the acquisition buttons has been clicked. Stops the function of the relevant acquisition button.

Active Camera section

Parameter	Description
Link Cameras	Only active if you have connected two structurally identical cameras to your system. **Activated:** Acquires images using two cameras in parallel. This is often the case with 2-channel images for ratio measurements or FRET measurements.
Active Camera	Shows the active camera. If you have several cameras connected, you can select the detector to use here.

Tools section

Depending on which modules you have purchased you see different tools available in this section, see Tools on Locate tab.

See also

Microscope Control Tool [666]

13.2.1.1 Configure your hardware setting favorites Dialog

Here you configure up to 20 new buttons to get quick access to your preferred camera and hardware settings.

Info

To create and edit settings you need the settings editor. Click on **Tools > Settings Editor**.

Parameter	Description
Favorite Settings	If you have not yet defined any buttons, you will see an empty list here. To create a new button, click on the **Add** button. Your Favorites are displayed as buttons on the **Locate** tab in the **Favorites** section. You can configure your favorite setting in the input fields:
- Name	Here you can enter a name for the button.
- Hardware Setting Ref.	Shows the selected hardware settings.
- Camera Setting Ref.	Shows the selected camera settings.
- Color	Here you can select a color for the related button. Click on the color dropdown list to choose a color.

Parameter	Description
- Use Color also for Button Text coloring	**Activated:** Uses the selected color as the button text color.
Available hardware settings on disc	Here you see a list of all hardware settings that are saved on your hard drive. Select the hardware setting that you want to use with the configured button. To add a hardware setting, simply drag & drop them to the desired button configuration.
Available camera settings on disk	Here you see a list of all camera settings that are saved on your hard drive. Select the camera setting that you want to use with the configured button. To add a camera setting, simply drag & drop them to the desired button configuration.

13.2.2 Acquisition Tab

Here you configure and control your acquisition experiments.

Info

The content of the tab changes depending on the configuration of your imaging system and the options that you activate or deactivate.
Settings that you configure in the top part of the tab have an effect on settings in the bottom part of the tab. Settings that you configure in the **Acquisition Parameter** tool group, e.g. in the **Channels** tool also apply to the acquisition of all images that you configure in the **Multidimensional Acquisition** tool group.

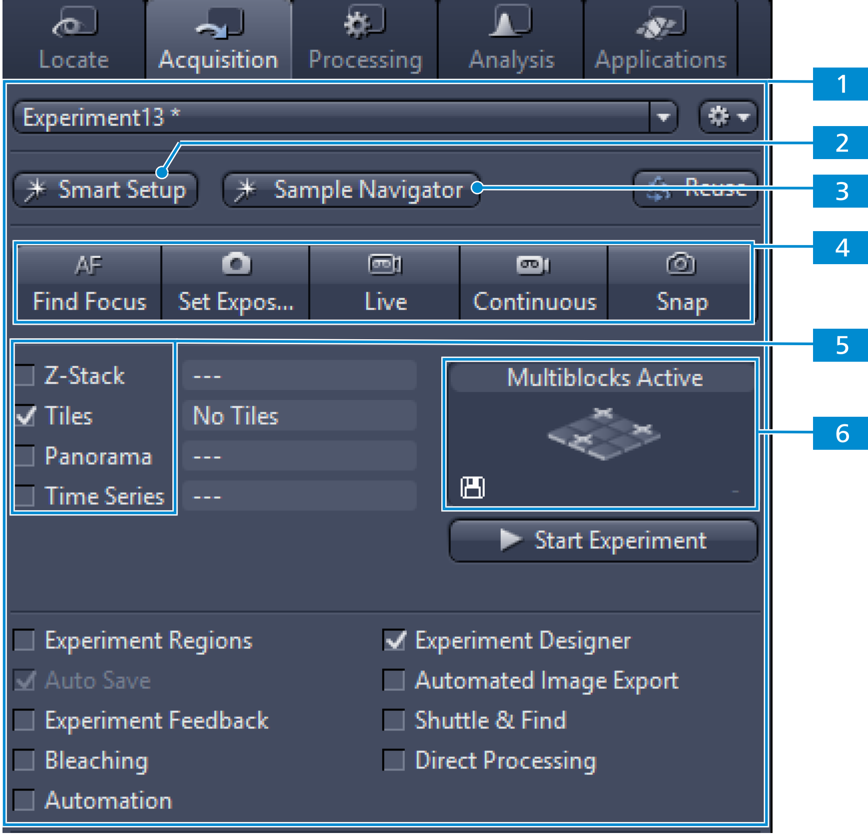


Fig. 52: Acquisition tab

1 **Experiment Manager**

The area above the blue tools where you can load and save your experiments, control acquisition and decide which tools will appear in the certain tool groups.

For further information on Experiment Options, see *Experiment Options [▸ 654]*.

2 **Smart Setup**

Opens the **Smart Setup** dialog, see Smart Setup

3 **Sample Navigator**

The **Sample Navigator** wizard is a tool to find the focus plane and quickly acquire an overview scan of your sample. You can also simplify the search for a region of interest for the actual imaging experiment. For further information, see *Using the Sample Navigator with LSM 980 and LSM 900 [▸ 69]*.

4 **Action Buttons**

With these buttons you control microscope and camera and acquire your images. For further information on action buttons, see *Action Buttons [▸ 653]*.

5 **Acquisition Dimensions**

Here you activate the desired dimensions (e.g. **Z-Stack** or **Tiles**) for your experiment. A drop down menu appears when selecting the dimensions to determine the sequence of acquisition. For more information, see *Acquisition Sequence [▸ 662]*.
The corresponding fields to the right of each dimension show a preview of how extensive the experiment will be (e.g. **9 Tiles**).
Below the dimensions section you activate additional experiment features (e.g. **Auto Save**) or special modules (e.g. **Shuttle & Find**).

6 **Experiment Preview**

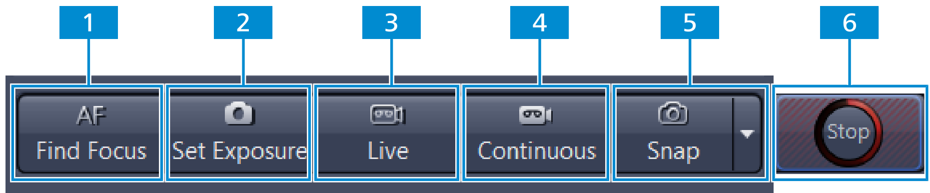
Here you can see a graphical representation of the configured experiment. The **Disc** icon indicates that you have enabled **Auto Save** function for the experiment.

See also

Smart Setup [▸ 655]

13.2.2.1 Action Buttons

With these buttons you control microscope and camera and acquire your images.
The Acquisition buttons on the **Acquisition** tab differ from the Acquisition buttons on the **Locate** tab.
The buttons on the Locate tab relate to an individual image. The buttons on the Acquisition tab relate to a multidimensional image with at least one channel.



1 **Find Focus**

Only visible if you have configured a motorized focus (MicroToolBox).
Starts an autofocus search using the settings from the **Focus Devices** tool. The autofocus search is performed for the selected reference channel in the **Channels** tool.

2 **Set Exposure**

Starts an automatic exposure time measurement with the settings defined in the **Light Path** and **Camera** tool.

3 **Live**

Starts the **Live Mode.** In the **Center Screen Area** you see the live image from the camera.

4 **Continuous**

Starts a series of **Snaps** using the settings defined in the **Light Path** and **Camera** tool. In contrast to a live image, the exact same camera setting that has been set in the **Camera** tool is used.

5 **Snap**

A so called "Snap" acquires a single image (snapshot).
For widefield systems an additional tiles snap option is available. You can choose between 2x2, 3x3, 4x4 and 5x5 presets.

6 **Stop**

Only active if one of the acquisition buttons has been clicked.
Stops the function of the relevant acquisition button.

13.2.2.2 Experiment Options

In the **Options** shortcut menu you can create new experiments and rename, save, import, export or delete existing experiments.

Menu item	Description
New	Creates a new, empty experiment. Enter a name for the experiment.
New from Template	Create a new experiment based on an existing experiment. The template experiment will not be modified. You can create your own template experiments by placing them in the folder **Carl Zeiss\ZEN\Templates\Experiment Setups**, which is located in the windows public documents folder and in the documents folder for each user.
Rename	Enables you to enter a new name for the experiment.
Save	Saves a modified experiment under the current name. An asterisk indicates the modified state.
Save As	Saves the current experiment under a new name. Enter a name for the experiment.
Reload	Reloads the selected experiment.
Set As Startup Default	Selecting this option will assign the currently loaded experiment as a default experiment, which is loaded every time the software is started. The startup default experiment is indicated by a special icon behind the experiment name. You can decide to start the software with a particular default experiment, with the last used experiment or with an empty new experiment in **Tools > Options > Startup/Shutdown > Experiment**.
Import	Imports an existing experiment. The experiment is shown in the **Experiment Selection** dropdown list.

Menu item	Description
Export	Exports the current experiment.
Delete	Deletes the current experiment.

13.2.2.3 Smart Setup

Smart Setup offers you support when configuring multichannel acquisition experiments. To start it click on the **Smart Setup** button on the **Acquisition** tab.

Select the fluorescent dyes and contrast techniques that you want to include in your experiment from a large dye database. Smart Setup takes the configuration of your microscope hardware and the properties of the selected dyes into account. Based on this information, it makes one or more suggestions for acquisition. You can adopt these into your experiment as required and make further changes to them there.

Info

If **Smart Setup** is unable to make a proposal, it is not possible to use the selected dyes, contrast techniques, or current microscope hardware to make acquisitions. Select other dyes or another contrast technique or configure your acquisition experiment using the **Acquisition Mode** tool and the **Channels** tool.

For working with Smart Setup please note the following:

- Smart Setup tries to configure the motorized components of your system for the acquisition of multichannel images.
- Smart Setup does not change any parameters of other acquisition dimensions (e.g. Z-stack, Time series, or Multi-position acquisitions).
- For widefield tracks it does not influence any camera parameters (e.g., Exposure time or Resolution).
- For LSM tracks it adjusts parameters within the Imaging Setup, the Acquisition Mode and the Channels tool windows.

Depending on your system you will see two buttons on top of the dialog.

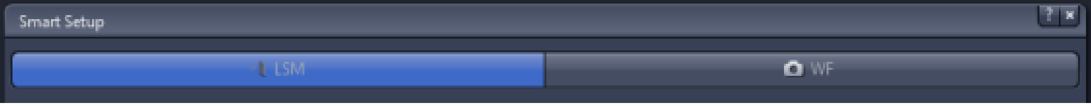


Fig. 53: Smart Setup Modes

If you select the **LSM** button you can use Smart Setup for configuring confocal experiments, see *Smart Setup (LSM) [▸ 658]*.

If you select the **WF** button you can use Smart Setup for configuring widefield experiments, see *Smart Setup (WF) [▸ 656]*.

13.2.2.3.1 Smart Setup (WF)

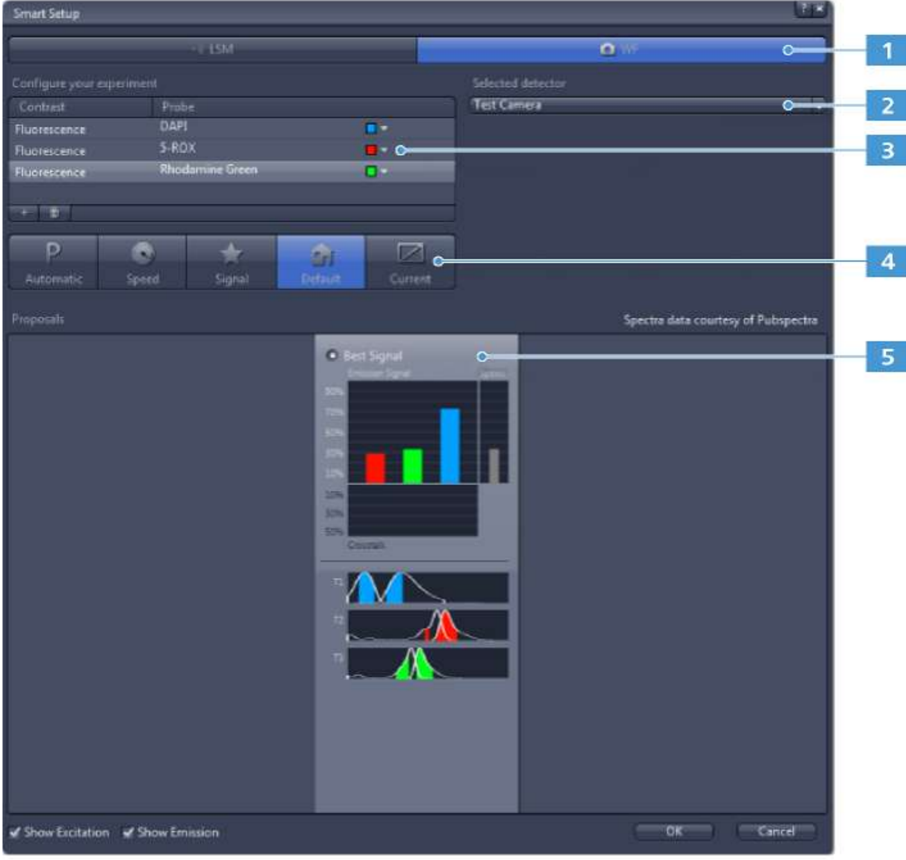


Fig. 54: Smart Setup (WF)

1	Imaging Mode Selection
2	Detector Selection
3	Experiment Configuration
4	Motif Buttons
5	Graphical Display of Proposals

Parameter	Description
Detector Selection (2)	Only visible if two or more cameras are configured for the system. Here you can select the desired camera for the experiment.
Configure your experiment (3)	Here you can add up to four reflected light fluorescence channels and one transmitted light contrast technique to your experiment. The added dyes or the contrast technique are shown in the list below. If you click on the **Add** button the *Add Dye or Contrast Technique [▸ 809]* dialog will be opened. There you can select the desired dye or contrast technique from the Dye Database.
Motif buttons (4)	Here you can optimize image acquisition regarding particular requirements like speed or quality. All parameters, e.g. camera resolution or dynamic range in the **Acquisition Mode** or the **Channels** tool, were set automatically. They will essentially influence the camera, detector, and lightning settings.

Parameter	Description
- Automatic	▪ The system will try to set the optimal resolution for the camera in the **Acquisition Mode** tool. The resolution will be calculated from camera parameters and numeric aperture; Microscanning will not be applied even if the camera supports this mode. ▪ The dynamic range for all fluorescence channels will be set to 50% or 80% for all transmitted light channels.
- Speed	▪ If binning is supported, one binning category will be set for the camera under the optimal resolution in the **Acquisition Mode** tool. ▪ Sets dynamic range of all fluorescence channels to 20% or 50% for transmitted light channels. ▪ Sets power of all Colibri-LEDs to 100%. ▪ Sets EMGain of the camera (if available) to half of Gain max. Sets read mode of camera to fastest. ▪ Creates an acquisition configuration which removes all reducers or neutral filters. ▪ Changes acquisition sequence for dimensions to fastest. Only effective with 2 or more dimensions.
- Signal	▪ Sets the optimal resolution for the camera in the **Acquisition Mode** tool. Microscanning will be applied if the camera supports this mode. ▪ Sets the dynamic range for all fluorescence channels to 90% or 100% for all transmitted light channels. ▪ Sets power of all Colibri-LEDs to 75%. ▪ Sets EMGain of the camera (if available) to 10% of Gain max. Sets read mode of camera to slowest.
- Default	Sets all parameters in **Channels** and **Acquisition Mode** tool to the default values. All changes will be overwritten and reset.
- Current	No changes are made. Only the necessary hardware settings for acquisition are applied by **Smart Setup**. **NOTICE! If you changed hardware settings in the Acquisition Mode tool manually and do not want to lose them, make sure you select the Current button.**
Proposals (5)	Here you can see the proposals made by **Smart Setup** displayed graphically. You can find a detailed description of the graphical display under *Graphical Display of Proposals [▸ 661]*. The proposals change the imaging settings in the **Imaging Setup** tool window accordingly. The number and type of proposals depend on the microscope hardware being used, the selected dyes, and the contrast technique:
- Best Signal	This proposal results in the best signal strength.
- Fastest	This proposal results in the fastest acquisition.
- Best Compromise	This proposal results in the best compromise between signal strength and fastest acquisition.

Parameter	Description
Show Excitation checkbox	Shows the excitation spectrum of the selected dyes in the graphical display.
Show Emission checkbox	Shows the emission spectrum of the selected dyes in the graphical display.
OK button	Adopts the proposal displayed as the current acquisition experiment. The suggestion overwrites existing experiments on the **Acquisition** tab.
Cancel button	Ends **Smart Setup**. The suggestions are not adopted into the experiment.

13.2.2.3.2 Smart Setup (LSM)

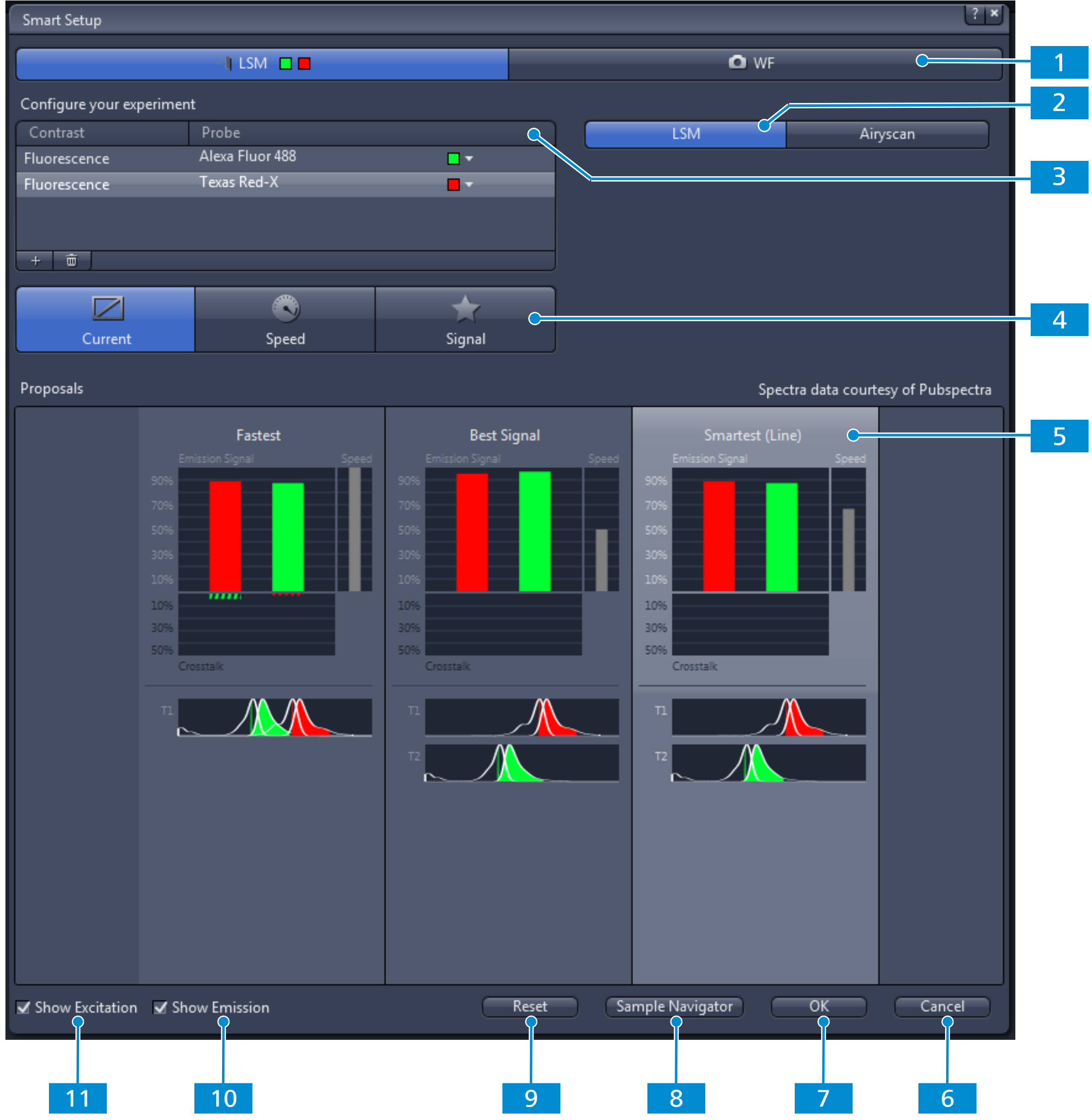


Fig. 55: Smart Setup (LSM)

No.	Parameter	Description
1	**Imaging Mode Selection**	Here you can select the **LSM** or **WF** imaging mode.
2	**Detection Mode**	If your system is equipped with an Airyscan detector, you can use **Smart Setup** to generate proposals for Airyscan instead of confocal acquisition, see also Airyscan Mode.
3	**Configure your experiment**	Here you can add up to 8 reflected light fluorescence channels and one transmitted light contrast technique to your experiment. The added dyes or the contrast technique are shown in the list. If you click on the **Add** button the *Add Dye or Contrasting Method Dialog [▸ 809]* dialog will be opened. There you can select the desired dye or contrast technique from the Dye Database.
4	**Motif buttons**	Here you can optimize image acquisition regarding particular requirements like speed or quality. If clicking on a button different parameters in the **Acquisition Mode** or the **Channels** tool, were set automatically. The automatic settings will influence parameters like Frame Size, Speed, Direction, Bit Depth (in Acquisition Mode tool) and Pinhole Diameter, Gain, Laser Power (in Channels tool), depending on the selected button. Various proposals for further experiment settings are shown in the graphical display below the buttons.
	- Current	No changes are made. Only the necessary hardware settings for acquisition are applied by **Smart Setup**. **NOTICE! If you changed hardware settings in the Acquisition Mode tool manually and do not want to lose them, make sure you select the Current button.**
	- Speed	▪ Sets the frame size to 400x400 pixels ▪ Sets the scanning speed to maximum value ▪ Sets the scanning direction to bi-directional ▪ Opens the pinhole to 2 Airy Units (AU)
	- Signal	▪ Aims to provide high quality images with best signal to noise ratio. ▪ Sets the frame size to a minimal value that fulfills the Nyquist criterion, but to a maximum of 2048x2048 pixel ▪ Sets the scanning speed to 6 ▪ Sets the scanning direction to uni-directional ▪ Sets the Bit Depth to 16 bit
5	**Proposals**	Here you can see the proposals made by **Smart Setup** displayed graphically. You can find a detailed description of the graphical display under *Graphical Display of Proposals [▸ 661]*. The proposals change the imaging settings in the **Imaging Setup** tool window accordingly.

No.	Parameter	Description
		The number and type of proposals depend on the microscope hardware being used, the selected dyes, and the contrast technique:
	- Fastest	This proposal results in the fastest acquisition.
	- Best Signal	This proposal results in the best signal strength and minimizes the level of cross talk.
	- Smartest (Line)	Combines the advantages of Fastest and Best Signal. It minimizes the number of tracks as well as cross talk.
6	**Cancel** button	Ends **Smart Setup**. The suggestions are not adopted into the experiment.
7	**OK** button	Adopts the proposal displayed as the current acquisition experiment. The suggestion overwrites existing experiments on the **Acquisition** tab.
8	**Sample Navigator** button	Opens the Sample Navigator, see Sample navigator LSM 980 and LSM 900.
9	**Reset** button	Clears the list of selected dyes and/or contrasting methods.
10	**Show Emission** checkbox	Shows the emission spectrum of the selected dyes in the graphical display.
11	**Show Excitation** checkbox	Shows the excitation spectrum of the selected dyes in the graphical display.

13.2.2.3.3 Graphical Display of Proposals

Info

The bars in the graphs only show relative values. The actual strength of the emission signal and the crosstalk in the image can deviate substantially from this estimate, as Smart Setup has no knowledge of the strength with which the sample has been dyed with the individual dye components.

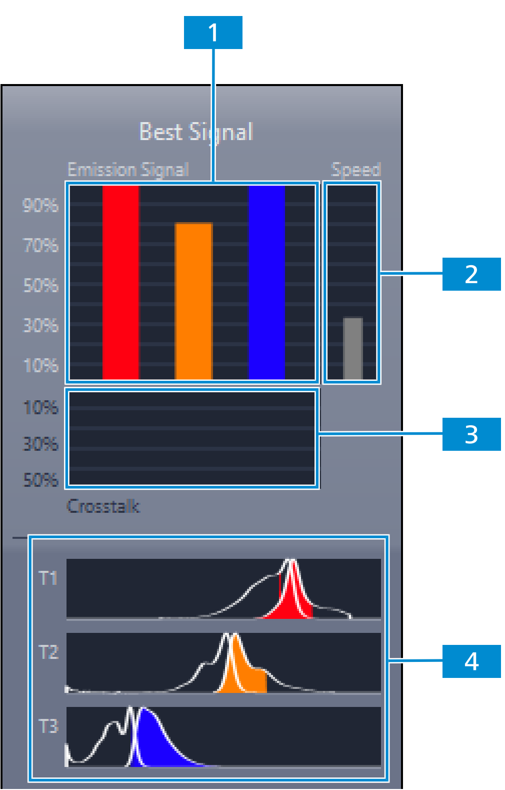


Fig. 56: Emission Signal, Speed, Crosstalk, and Tracks

1 Emission Signal
A filled, colored bar in the **Emission Signal** display field shows the relative emission signal to be expected for the corresponding channel. The channel color corresponds to the color of the selected dye in the **Configure Experiment** section.

2 Speed
A gray bar in the **Speed** display field represents the approximate acquisition speed that can be expected. This is the time required for the movement of microscope hardware during multichannel acquisition. Camera exposure times or parameters for other acquisition dimensions are not taken into account here.

3 Crosstalk
A hatched bar in the **Crosstalk** display field shows the expected relative crosstalk originating from one or more dyes for other channels.

4 Tracks display

Only visible if the **Show Excitation** and / or **Show Emission** checkboxes are activated.

The various tracks are labeled with **T1**, **T2** etc.. The white lines show the excitation and emission spectra of the dyes schematically. The spectra are filled in color in the places that will be acquired by the acquisition configuration suggested by **Smart Setup**. Transmitted light channels are displayed as a white field.

13.2.2.4 Reuse

The Reuse functionality is only available if you have loaded an image in *.CZI image format. Then, the **Reuse** button will then appear on the **Acquisition** tab. Otherwise, the **Reuse** button is not active.

With this function you can apply the experiment setup of the acquired image to the current experiment. This will help you to easily reproduce the acquisition conditions for the next image. The function only works correctly if the system configuration at the time of acquisition is identical to the system configuration at the time when you execute the function.

Removing components (e.g. filter cubes, LEDs, cameras, etc.) can result in an experiment being created incorrectly. It is therefore essential that you check after executing the Reuse function whether the configuration of the experiment is in line with your expectations.

Using the Reuse function for a Z-stack promts a confirmation asking whether to place the Z- stack at the current focus position or the original focus position of the acqired image.

Note, that the original position may be way off the current position and starting an experiment right away can lead even lead to the destruction of your sample.

Info

Clicking on the **Reuse** button overwrites the current experiment without a prompt and marks it as having been modified. This can be seen from the appearance of an asterisk after the file name. If you want to keep the experiment in its previous form, you must save the modified experiment with a new file name under **Experiment Manager | Options | Save As**.

If you acquire images and save them in *.CZI image format, the following acquisition conditions are saved together with the image:

- Information on the type and status of your imaging system
- Time of acquisition
- Parameters set in the software.

13.2.2.5 Acquisition Sequence

The available options in the dropdown list depend on the selected acquisition dimensions.

Acquisition dimensions	Selection option	Function
Z-Stack	**Full Z-Stack per Channel**	Acquires the complete Z-stack for a channel and then processes the next channel.
	All Channels per Slice	Acquires all channels for each Z-plane and then locates the next plane of the Z-stack.
Z-Stack **Tiles**	**Full Z-Stack per Channel**	Acquires the complete Z-stack at a tile/position for each channel and then processes the next channel. Once all Z-stacks at a tile/position have been acquired, the next one is located.

Acquisition dimensions	Selection option	Function
	All Channel per Slice	Acquires all channels at a tile/position for each Z-plane before locating the next plane of the Z-stack. Once all Z-planes and channels at a tile/position have been acquired, the next one is located.
Z-Stack **Time Series**	**Full Z-Stack per Channel**	Acquires the complete Z-stack for each channel for a time point and then processes the Z-stack of the next channel.
	All Channels per Slice	Acquires all channels for each Z-plane for a time point before locating the next plane of the Z-stack.
Tiles **Time Series**	**All Tile Regions per Time Point**	Acquires images for all channels as well as images at all positions of the experiment for each time point and then waits for the next time point.
	Time series per tile	Acquires the complete time series at a position with all channels, before doing the same at the next position. Connected tile images are, however, acquired in full for each time point.
Z-Stack **Tiles** **Time Series**	**Time-Regions-Tiles-Channels-Z**	Acquires images for all channels as well as images at all positions of the experiment for each time point and then waits for the next time point. The complete Z-stack is acquired at every position for each channel and the next channel is then processed.
	Time-Regions-Tiles-Z-Channels	Acquires images for all channels as well as images at all positions of the experiment for each time point and then waits for the next time point. Acquires all channels for each Z-plane and then locates the next plane of the Z-stack.

Acquisition dimensions	Selection option	Function
	Regions-Time-Tiles-Channels-Z	Acquires the complete time series at a position with all channels, before doing the same at the next position. Acquires the complete Z-stack for each channel and then processes the next channel.
	Regions-Time-Tiles-Z-Channels	Acquires the complete time series at a position with all channels, before doing the same at the next position. Connected tile images are, however, acquired in full for each time point. Acquires all channels for each Z-plane and then locates the next plane of the Z-stack.

13.2.3 Processing Tab

Here you apply processing functions to acquired or loaded images. For general information of the image processing, see *Image Processing Workflow [▸ 90]*. For the detailed descriptions of the functions and the processing workflow please read more under Image Processing Functions.

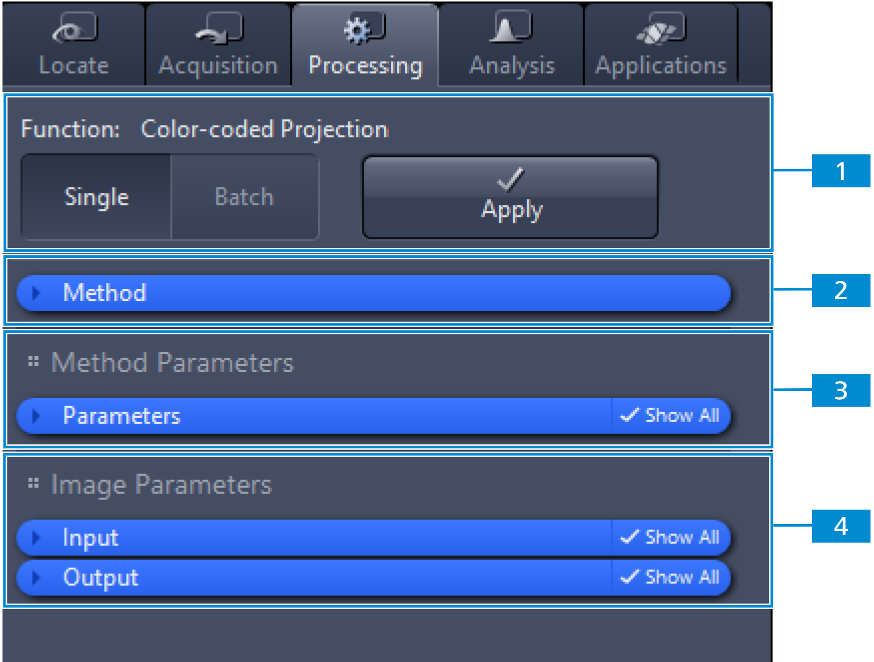


Fig. 57: Processing Tab

1 **Function**
Using **Single** processing, you can apply a selected processing method, with the relevant method and image parameters, to a single image.
Using **Batch** processing, you can apply a selected processing method, with the relevant method and image parameters, to a list (batch) of images. In this mode only a limited selection of processing functions is available. For more information, see *Applying Batch Processing [▸ 102]*.
With the **Apply** button you apply the selected method to the input image.

2 **Method**
Here you select the image processing functions. Open the **Method** tool to show the list of IP functions.

3 **Method Parameters**
Here you configure the parameters of the selected image processing function. Click on the **Parameters** tool to show the parameters of the selected IP function.

4 **Image Parameters**
Here you configure the image parameters of the input and output image. Click on the **Input** tool or **Output** tool to open input/output image settings. For more information, see *Image Parameters - Input Tool [▸ 769]* and *Image Parameters - Output Tool [▸ 769]*.

13.2.4 Analysis Tab

Here you find different tools available for image analysis.

See also

- Interactive Measurement Tool [▸ 770]
- Image Analysis Tool [▸ 775]
- Intellesis Trainable Segmentation Tool [▸ 442]

13.2.5 Applications Tab

On this tab you have the following tools available:

- *Guided Acquisition Tool [▸ 366]*
  for creating an automated workflow to acquire images (overview), detect relevant objects (image analysis) and re-image these positions with another experiment.
- *Topography Tool [▸ 265]*
  for acquiring or loading an existing confocal z- stack and exporting it to ZEISS ConfoMap for further processing.
- *Layer Thickness Measurement Tool [▸ 267]*
  for acquiring or loading an existing confocal z- stack and perform layer thickness measurements.
- *Automated Photomanipulation Tool [▸ 255]*
  for creating an automated workflow to acquire multi-position images, determine relevant ROIs for photomanipulation (image analysis), and execute the photomanipulation experiment at these ROIs.
- *Direct Processing Tool on Processing Computer [▸ 354]*
  for ensuring that the processing computer reads incoming files and starts the processing in case of remote processing.
- *arivis Vision4D Tool [▸ 797]*
  for sending an image to the arivis Vision4D software and open it with an analysis pipeline.
- *APEER [▸ 244]*
  for downloading an APEER module and using it locally in ZEN.

13.2.6 Extensions Tab

Our extensions concept allows to extend ZEN basic functionality by implementing third party extensions, e.g. ImageJ. The extensions concept is a part of **OAD (Open Application Development)** for ZEN (see also Open Application Development (OAD)).

Depending on which extension you have activated, you will see the extension's functions and controls on the **Extensions** tab. Please notice that we will not describe any functions of third party extensions here. Therefore use the third party documentation for each extension.

You can find more information on OAD and the supported extensions under **www.zeiss.com/zen-oad**.
For more information on ImageJ, see *ImageJ [▸ 411]* .

13.3 Tools

13.3.1 Tools on Locate Tab

13.3.1.1 Microscope Control Tool

The configuration of your system according to your MicroToolBox (MTB) configuration setting is shown here. A valid microscope configuration has to be created first using the MTB2011 Configuration program. The light path follows the light starting with the light source to the specimen and from there to the camera or eyepiece. It displays control elments for all motorized and manually operated components. Here you can interactively adjust the microscope and its components.

Info

If you are not using any motorized components, you will have to make the relevant adjustments manually.

Keep the following points in mind when working with this tool:

- To adjust a component, left-click on the relevant icon.
- Icons with an **arrow** symbol in the bottom right corner contain dialogs that allow you to configure additional settings. To open the dialogs, left-click on the corresponding icon.
- Icons with a **hand** symbol in the bottom left corner indicate components that have to be operated manually.

Icon	Parameter	Description
	Eyepiece	Displays the total magnification of the selected beam path with all activated intermediate magnifications above the **Eyepiece** icon. To direct the light path fully to the eyepiece, simply left-click on the icon.
Closed / Open	**Shutter**	Here you can set the shutter to **Open** or **Closed**. The status is displayed in text form above the icon.

Icon	Parameter	Description
	Reflector Turret	Here you can select one of the configured filter cubes for reflected light techniques from the list.
	Nosepiece / Objective	Select the desired **Objective** from the list.
	Stage	Here you see the options for Stage Control and Focus Control. ▪ **Stage >>:** Opens the *Stage Tool [▸ 798]* in the **Right Tool Area**. There you can move the microscope stage virtually with the help of a software joystick or by entering absolute coordinates. You can also calibrate the stage within that tool. ▪ **Focus >>:** Opens the *Focus Tool [▸ 800]* in the **Right Tool Area**. There you can move the focus drive virtually with the help of a software joystick or by entering absolute coordinates. You can also calibrate the focus drive within that tool.
	Aperture Diaphragm	Adjust the diaphragm opening (0% to 100%) using the slider or spin box/input field.
	Filter Wheel	Here you can enter the first neutral density filter (e.g. 0.4%, 6%, 100%, 100%) that you require.
	Condenser	The condenser is only available in the **Transmitted Light** path. Select the contrast method from the list (e.g. brightfield, darkfield, phase contrast ring 1, 2, 3, DIC I, II, III).
	Camera/ Eyepiece Switch	Select whether you want to direct the light to the camera only (**100% Camera**), to the camera and the eyepiece (**30% Eyepiece/70% Camera**) or to the eyepiece only (**100% Eyepiece**) from the list .
	Camera	Shows the selected camera above the icon.
	Microscope Manager	Opens the *Microscope Manager [▸ 668]* dialog.
	Reflected Light/ Transmitted Light Switch	If your microscope has a halogen lamp for both reflected and transmitted light illumination, here you can select whether you want to control the halogen lamp for reflected light illumination or the halogen lamp for transmitted light illumination.
QFT 400	6x Motorized Beam Splitter Wheel	This device is part of the Motorized Dual Filter Wheel. Select the desired Dichroic position from the dialog. Switching time is about 300 msec between neighboring positions.

Icon	Parameter	Description
	6x Motorized Emission Filter Wheel	This device is part of the Motorized Dual Filter Wheel. Select the desired Emission Filter position from the dialog. Switching time is between 60 and 240 msec between neighboring positions (depending on the speed configuration in the MTB2011 Configuration program).
	6x Motorized Excitation Filter Wheel	Select the desired Excitation Filter position from the dialog. Switching time is between 70 and 300 msec between neighboring positions (depending on the speed configuration in the MTB2011 Configuration program).
	12x Motorized vReflector Changer	If a Motorized Dual Filter Wheel and a Motorized Emission Filter Wheel is present, up to 12 virtual Reflektor positions can be configured in the MTB2011 Configuration program. Select the desired filter combination from the list of available positions. This is more convenient than adjusting excitation, dichroic and emission filters individually.

13.3.1.1.1 Microscope Manager

Parameter	Description
Contrast Manager	
Mode	Select the setting for the contrast mode from the **Mode** dropdown list.
- Off	The Contrast Manager is not used. All settings must be made manually or via a settings file.
- On Demand	The function of the Contrast Manager is activated via the touch screen on the microscope.
- Contrast Retaining	If core components (e.g. condenser, reflector, shutter) for a certain contrast technique are changed, dependent components are also changed accordingly.
Method	Select one of the available methods for the contrast mode here.
Light Manager	
Enabled	**Activated:** Activates the Light Manager. Activates the **Mode** dropdown list in the Light Manager.
Mode	Select a setting for adjusting the brightness of the light here.
- Objective	Adjusts the brightness of the light via the lamp voltage. The color temperature changes accordingly.
- Classic	Adjusts the brightness on the basis of the available filter wheels. The color temperature is retained. Only if the brightness adjustment cannot be achieved via the filter wheels does adjustment take place via the lamp voltage.
Dazzle Protection	**Activated:** Activates dazzle protection. Dazzle protection prevents light from passing through the eyepiece and dazzling the user, for example when reflector positions are

Parameter	Description
	changed. This is mainly achieved by closing the reflected or transmitted light shutter. If no shutters are installed, the lamp voltage is adjusted.
Parfocal Correction	**Activated:** Parfocal correction is activated.
Parcentral Correction	**Activated:** Parcentral correction is activated.

13.3.1.2 Camera Tool

Here you can configure all the settings for the selected camera.

Please note that the functions and settings in this tool depend on which camera you are using meaning not all cameras have all the functions described in here.

13.3.1.2.1 Exposure Time Section

The **Exposure Time** section enables you to control the exposure settings of your camera.
If you use automatic exposure, you can select an area on the camera sensor which is used to calculate the exposure time.

Parameter	Description
Time	Specifies the duration of the image acquisition. Selects the unit of time (min, ms, s, µs).
Auto Exposure	**Activated:** The exposure time is calculated automatically every time an image is acquired. The exposure time in the corresponding input field fluctuates accordingly. **Deactivated:** The exposure time must be set manually.
Intensity	Enables you to compensate for underexposure or overexposure if you are not content with the auto exposure result. ▪ 5% - 100%: Darkens the image (compensates for overexposure) ▪ 100% - 200%: Brightens the image (compensates for underexposure)
Set Exposure	Starts a one-off measurement of the exposure time, which is then used for all subsequent images. Deactivates the **Auto Exposure** checkbox. If you are not satisfied with the result, you can adjust the measured exposure time manually.
Spot Meter / Focus ROI	**Activated:** The exposure time and focus measurements use the intensity values within a specified area instead of the entire camera sensor area. This improves the results for the area to be acquired. If the red **Spot Meter / Focus ROI** frame is not visible in the live image, right-click in the live image and select **Spot Meter / Focus ROI** from the context menu.

Parameter	Description
Binning	Here you can set the binning. Binning combines the information of neighboring camera pixels into a single larger pixel. For example, if the binning is set to 2 × 2, four pixels are combined to one. Increasing the binning means weaker signals can be detected for a given exposure time. For CCD cameras, binning increases sensitivity by improving the signal-to-noise ratio, with resolution being decreased by the same factor. In the case of CMOS cameras, only the signal intensity is increased and the pixel count and resolution gets reduced correspondingly.
Binning-independent Brightness	Only visible when the **Show all** mode is activated. Because **Binning** generally increases signal intensity, the brightness of the image normally also increases correspondingly. By activating this checkbox, the brightness level is automatically fixed (depending on the camera, either through exposure time adjustment or averaging), no matter the binning setting.
IP Quality	Here you can select the color interpolation quality (**IP Quality**) for the acquired image. Please note that this function does not apply to **Live** mode. **Fast**: color interpolation for optimum speed (shorter computation). **High**: color interpolation for optimum quality (less artifacts). This mode is only effective with binning factor 1.
Subsampling	Here you can reduce the amount of data acquired to achieve faster frame rates. By subsampling 2x2, the effective pixel pitch is increased by sampling only every other pixel, thus reducing the overall data size of your image. The modern CMOS sensors in the Axiocam 705 and 712 offer a on-chip subsampling mode. This subsampling mode enables a dramatic increase in frame rate especially for time series acquisition (at short exposure times) at an full field of view of the camera. In addition, the amount of produced image data is decreased. As this is done by skipping every second pixel in x and y direction, the optical resolution is decreased accordingly. Especially in high magnification objectives at lower NA values, the image resolution is optically limited. So this function can be used to minimize empty image information.
Resolution	Displays and selects the camera resolution, e.g. 1024 x 1024 px.

13.3.1.2.2 White Balance Section

This section is only visible if you are using a color camera.
The section enables you to adjust the color balance to a neutral hue independent of the light source used.

Save suitable white balance settings using the **Settings** section to ensure color reproducibility of images acquired in the future.

Parameter	Description
Auto	Compensates for the color temperature of the light source automatically to yield a neutral hue. The entire camera sensor area is measured. If there are no pure white areas on the sample and **Auto** does not yield the desired results, measure and compensate for the color temperature of the light source as follows: ▪ Transmitted light: Move the sample such that a clear and transparent region is illuminated or remove the sample from the microscope. Click the **Auto** button to perform the auto white balance. ▪ Reflected light: Use a neutral surface (e.g. a piece of white paper) as a sample. Click the **Auto** button to perform the auto white balance. You can now acquire white balanced images of your sample with the above settings.
Pick...	Enables you to select a reference pixel for white balance from the live image. The selected pixel should be neutral white.
3200 K	Applies a predefined color balance setting to compensate for the color temperature of a halogen light source at approximately 3200 K.
5500 K	Applies a predefined color balance setting to compensate for the color temperature of an LED light source at approximately 5500 K.
Show Channels	Enables you to set the color balance of each color channel (red/cyan, green/magenta and blue/yellow) individually to make the image appear neutral.
Color Temperature	Changes the overall color temperature of the image from cool (blue cast) to warm (red cast). The color channels (red/cyan, green/magenta and blue/yellow) are adjusted automatically. The **Color Temperature** setting can work against the settings applied using **Show Channels**. Use **Color Temperature** for fine tuning in combination with **Pick...** if **Pick...** does not give perfect results.
Saturation	Changes the colorfulness of the image.
Reset	Resets any color changes and sets the white balance value to 6500 K.

13.3.1.2.3 Acquisition ROI Section

In this section you can define a **Region Of Interest (ROI)** on the camera sensor which will be used for acquisition. A smaller ROI can increase the acquisition speed.

The region of interest is indicated by a blue frame in the preview window and can be moved and resized freely. The preview window always shows the entire camera sensor area which can be acquired.

The **Pixel Size** shown below the preview window indicates the size in µm to which a pixel corresponds. This depends on the camera sensor properties and on the binning.

Parameter	Description
Maximize	Selects the entire available image sensor area as the region of interest.
Center	Positions the region of interest precisely at the center of the image.
Size	Sets the width and height of the region of interest in pixels.
Offset	Specifies the position of the top left corner of the **Acquisition ROI** (blue frame) with respect to the top left corner of the preview window.
Refresh Overview	Acquires and displays an image in the preview window with the current ROI settings. This has no effect on the image in the **Center Screen Area**.
Crop	Allows you to specify a ROI (Region of Interest) in a snapped image. The "cropped" area is used as ROI for the next image acquisition. If no image is available in the **Center Screen Area** the button is deactivated.

13.3.1.2.4 Gain Section

Using the gain adjustment amplifies the signal intensity and brightness of the camera image while at the same time reducing the available dynamic range.

For Axiocam 702, 705, and 712 models: The Gain mode allows an signal amplification as specified in the camera data sheet. For these CMOS sensor based camera models, also the sensitivity of the cameras is amplified and the cameras can detect weaker signals. Please note, that by amplifying a signal, the max/min signal intensity which can be acquired will be limited. The consequence is a reduction of the available dynamic range. Gain 4 (opt) is a best compromise between sensitivity and available dynamic range. Gain 1x (min) is the minimum signal amplification with the highest dynamic range. Gain 16x (max) is the highest sensitivity with the smallest dynamic range.

HDR (0.2x) Mode

The High Dynamic Range Mode of the 702, 705, 712 cameras offers a method to increase the available dynamic range by a factor of 5x. This methods returns noise free information in very dim image areas and can record simultaneously very bright image areas. As the additional intensity range is extended for brighter image signals, regular signals get 5 times dimmer (0.2 times) compared to a standard setting. This is achieved by combing two images with different adjustments: one at a shorter exposure and a low gain value for the bright signals and one image at longer exposure times and higher gain for the weak signals. The used exposure times are automatically derived from the current camera setting. It also works in the live image mode, but reduces the frame speed accordingly.

Please execute a proper exposure readjustment after activating this feature. This feature is very useful if the camera should acquire reflective surfaces, i.e. in materials applications, or in polarization microscopy of minerals.

13.3.1.2.5 Post Processing Section

Here you can apply basic image processing functions while acquiring the image. This can be helpful if certain image processing steps are necessary for any acquired image and saves image processing work later in a job.

Depending on the camera model, different settings are available.

Parameter	Description
Black Reference	Influences the live image and each image acquired. For the black reference to work, you first need to acquire a reference image. Define a corresponding reference image using the **Define** button. **Activated:** Applies the measured black reference to the image. **Deactivated:** The measured black reference is not used. The reference image is retained. If longer exposure times are used (from exposure times of approx. >5s, depending on the camera), individual bright pixels may become visible with CCD or CMOS sensors. With the help of the black reference these effects are measured and corrected in accordance with the exposure time employed. It is recommended that you repeat this measurement at certain intervals. This correction is recommended in particular for applications that involve long exposure times, i.e. for which very little light is used (live cell imaging, fluorescence images). The availability of a black reference for the selected camera can be checked on the menu **Extras** | **Calibration Management**.
- Define	Automatically defines the black reference. The measurement lasts for several seconds. The **Black Reference** checkbox is then activated automatically.

Info

For the measurement the camera must see a completely dark image. The light path to the camera must therefore be blocked. Set the eyepiece switch to 100% eyepiece and close the reflected light/transmitted light shutter. To define the black reference, click on the **Define** button.

Parameter	Description
Shading Correction	Shading correction is used to correct optical effects, such as minor differences in illumination or static contaminants in the beam path, with the help of a reference image. The reference image must be acquired without a sample. You can select between two modes **Global** and **Specific** from the dropdown list (see description below). After you have selected the mode simply click on the **Define** button. **Activated:** Applies the defined shading correction to the image. The applied correction mode is multiplicative. **Deactivated:** The measured shading correction is not used. The reference image is retained.
- Define	Automatically calculates the shading correction.
- Global	Performs an objective specific shading correction. This is the default method for shading correction. The following components will be considered: ▪ Magnification: Objective and Optovar ▪ Camera bit depth and RGB/BW mode ▪ Camera type and port position Fluorescent filters or other fluorescence specific components are not considered. In principle, shading correction is objective specific. A separate reference image has to be created for each objective. Once calibration has been completed, the correction image associated with the objective being used is loaded automatically if shading correction is active. If no correction image is available for an objective, the **Shading Correction** checkbox is automatically deactivated when the objective in question is swung in. Objective recognition on a motorized or encoded microscope is required for these automatic actions.
- Specific	Performs channel-specific shading correction. In this case the fluorescence filter block used is saved with the shading file. If the fluorescence channel is changed, a previously created reference image is also loaded. The availability of created reference images can be checked on the menu **Tools** | **Calibration Manager**. Incorrect reference images can also be deleted there. The following components will be considered: ▪ Contrasting method and condenser ▪ Fluorescence reflector and beam splitter ▪ Spinning disc fluorescence filter

Info

An empty image without structures at a medium illumination intensity is required for the shading correction measurement. To create this image, locate an empty position on the slide outside the sample and acquire an image for shading correction there. There must be no visible structures on the slide, as these will be incorporated into the correction image and could then lead to a visible artifact at other positions. It may be necessary to clean the slide and defocus the microscope slightly. You should bear in mind that Köhler illumination needs to be set correctly. No part of the image must be overexposed.

Parameter	Description
Enable Noise Filter	**Activated:** Filters the noise in the acquired image according to the adjusted threshold. Affects acquired images only. The live image does not change. Deals with sporadic bright events in single pixels on CMOS sensors, like blinking pixels in dark images or spurious cosmic ray events. This feature is most useful for situations with high gain settings for acquisition of very dim signals with CMOS or EMCCD cameras. In combination with a correct black reference an absolutely flat signal background can be produced.
- Threshold	The higher the value, the greater the tolerance for noise. The lower the value, the stronger the noise reduction. The noise filter reduces the extent to which individual pixels deviate from the average value of their nearest neighbors. The **Threshold** corresponds to a tolerance value. If the deviation of the middle pixel value from the average value of the pixels immediately surrounding it exceeds the tolerance value (i.e. it is interpreted as noise), it is replaced by the average value. This technique reduces the noise of individual pixels that are produced, in particular with EMCCD cameras and CMOS cameras. The selected technique prevents any changes being made to object edges, as in most cases these are larger than individual pixels. This filter is also suitable for removing individual "hot pixels" from an image without having to acquire a reference image in advance.
Enable Unsharp Mask	Enhances contrasts at fine structures and edges. Thus, the resulting image appears clearer and enriched in detail.
- Strength	Controls the amount of contrast enhancement applied to fine structures and edges. The higher the strength, the darker or lighter the resulting edges, compared to the original image.
- Radius	Determines the size of detail to be enhanced. A small radius enhances smaller details. The radius also affects the appearance of enhanced edges. A large radius leads to a visible halo along enhanced edges. The larger the radius, the broader the halo.
- Color Mode	Determines the calculation method, which affects the appearance of the output image. ▪ **RGB**: – The **Unsharp Mask** filter calculates the sharpness for each color channel individually. – The color saturation and the color of structures may be changed and color noise may occur. ▪ **Luminance**: – The **Unsharp Mask** filter calculates the sharpness based on the luminance signal computed from the RGB channels. – This mode avoids possible color noise or shift in color saturation, which could be induced by certain image textures.
- Auto Contrast	**Activated:** Enables you to adjust the **Contrast Tolerance** (0-20). **Auto Contrast** only works in RGB color mode.

Parameter	Description
- Contrast Tolerance	Increasing the contrast during unsharp masking is achieved by broadening the distribution of intensities. This corresponds to a spread of the image histogram. Controls how much the intensity distribution is spread and thus how strong the contrast is increased. ▪ **Contrast Tolerance = 0** : No spread of intensities, no increase of contrast ▪ **Contrast Tolerance = 20**: Maximum spread of intensities, maximum increase of contrast
- Clip To Valid Bits	**Activated:** Composes the processed image of the same colors as the original image (i.e. the value range of the output image is adjusted to the color range of the input image). **Deactivated:** Colors not present in the original image may appear in the processed image.

See also

Shading Correction [▸ 120]

13.3.1.2.6 Settings Section

In this section you can save all the settings you have made in the camera tool to a settings file (*.**czcs**). This is very helpful because you can restore/load your saved settings very quickly when starting the software again.

Parameter	Description
Default	Resets all camera settings in the **Camera** tool to the factory default settings. These settings can also be selected from the dropdown list of available camera settings to the right of the **Default** button. To do this, select the **Original Settings** entry.
Options	Opens the *Options [▸ 676]* menu.
Reload	Undoes the changes you have made to a loaded setting and restores the original status of the loaded setting.

13.3.1.2.6.1 Camera Tool Options

Parameter	Description
New	Creates a new camera setting. Enter a name for the camera setting in the input field. To save the camera setting, click on the **Disc** icon to the right of the input field.
Rename	Renames the current camera setting. Enter another name for the camera setting in the input field. To save the camera setting, click on the **Disc** icon to the right of the input field.
Save	Saves the current camera setting.

Parameter	Description
Save As	Saves the current camera setting under a new name. Enter a new name in the input field. To save the camera setting, click on the **Disc** icon to the right of the input field.
Import	Imports an existing camera setting.
Export	Exports the selected camera setting.
Delete	Deletes the selected camera setting.

13.3.1.2.7 Mode Section

In this section you can adjust how the software retrieves the camera sensor data.

Parameter	Description
Color Mode	This parameter is available for color cameras only.
– RGB	Transmits the image data of a color camera unchanged. This corresponds to the standard operating mode of a color camera.
– B/W	Treats the image data of the color channels as grayscale. The data of related color channels are averaged. The saturation of the camera appears reduced as a result. This process does not change the spectral properties of a color camera. The image information of the color sensor still undergoes color interpolation. An infrared filter also restricts the spectral sensitivity of the color camera compared to the spectral sensitivity of a genuine black and white camera.
Live Speed	Here you can select the live image update speed (For CCD based Axiocam models = slow, medium, fast). Enables you to focus or to find regions of interest on a sample quickly. A high live image update speed reduces the exposure time of the live image, even at longer exposure times used for image acquisition. To achieve a similar impression of image brightness, however, the image data supplied must be adjusted digitally, which may generate a certain amount of noise or reduce the resolution of the live image. For CMOS sensor based camera model (e.g. 712 / 705) High Res, Fast, Low Light).
– Fast	Represents the image optimized and requires a shorter computation time.
– High	Represents the image without artifacts and with a higher image quality. This mode is only effective with binning factor 1.

Parameter	Description
Bit Depth	The **Bit Depth** function enables the reduction of the delivered camera bit depth. It is translating the 14 bit based camera data values into a smaller value of 12 bit or 8 bit. This has an visible effect of the number range in the image histogram, the dynamic range and the corresponding image file size in an uncompressed CZI image. As default, the Axiocam models deliver 14 bit per pixel (Axiocam 503/ 506/ 512/ 702) and 12 bit/pixel in case of the Axiocam 305. 14 bit and 12 bit per pixel values need to be stored in two bytes in an CZI image file. In case of an translation to 8 bit per pixel, only one byte is needed. Therefore it is possible to reduce the produced image file size by a factor of two. This is especially beneficial, if large amount of image data is acquired. By reducing the native camera bit depth to smaller values, the available number range for the digital image signal is decreased. Therefore, the available amount of resolvable intensities in one image scene is decreased accordingly.
– LUT min/LUT max (only available for 8 bit mode)	LUT stands for Look Up Table. It describes the translation method for quickly translating digital numbers in a different range. If the full swing of the input signal is not used, the reduction of bit depth can be adopted accordingly by the translation starting point "Lut min" and the translation end point "Lut max". If the used intensity range equals just an 8 bit value range, no information is lost and the unused bits can be excluded from being stored by an suitably adjusted translation table. Available range is 0 to 1. The value 0 equals 0% of the input range. The number 1 equals 100% of the input range.
– Gamma (only available for 8 bit mode)	The 14 bit to 8 bit translation is linear by default, which equals a Gamma value of 1. By assigning values larger or smaller then 1, the translation becomes non linear. Values <1 selectively reduce dim signal intensities. Values >1 selectively amplify dim signal intensities.

13.3.1.2.8 Trigger Control Section

Only visible if the selected camera has a trigger input/output.

Trigger Out section

Using the trigger output you set how the camera sends a trigger signal to an external component (e.g. shutter).

Info

Activate both checkboxes if you want the trigger signal to be generated both during the live image and during acquisition.

Parameter	Description
Enable for Snap	**Activated:** Generates the trigger signal during the acquisition of an image.
Enable for Live	**Activated:** Generates the trigger signal during the live image.

Parameter	Description
Control Signal	
- Active High	The **Control Signal** jumps from 0 Volts to 5 Volts when the camera's exposure begins. Following exposure it returns to 0 Volts.
- Active Low	The **Control Signal** jumps from 5 Volts to 0 Volts when the camera's exposure begins. Following exposure it returns to 5 Volts.
Shutter Open Delay	Here you can enter the delay before acquisition.

Trigger In section

The trigger input allows you to trigger acquisition by the camera using an external trigger signal.

Info

Due to its inertia, a mechanical shutter needs a certain amount of time to change from the closed to the open position after the control signal has been generated. To ensure that this transitional state is not recorded during the exposure of the sensor, the start of actual acquisition can be delayed by an adjustable period of time.

Parameter	Description
Enable for Snap	**Activated:** Only acquires the image after the trigger signal has been received.
Control Signal	
- Active High	The **Control Signal** jumps from 0 Volts to 5 Volts when the camera's exposure begins. Following exposure it returns to 0 Volts.
- Active Low	The **Control Signal** jumps from 5 Volts to 0 Volts when the camera's exposure begins. Following exposure it returns to 5 Volts.

13.3.1.2.9 Model Specific Section

Only visible if the **Show All** mode is activated.

To show the section in full, click on the **arrow** button .

In this section you see additional, model-specific camera settings depending on which camera you use on your system.

Reset button

Resets all entries to the original values.

13.3.1.2.9.1 Axiocam 506

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 90 CW**
- **Rotate 90 CCW**
- **Rotate 180**
- **Mirror at +45 Diagonal**
- **Mirror at -45 Diagonal**

Acquire section

Readout Speed (MHz)

Readout speed can be varied between 39 MHz and 13 MHz if the camera is operated on the USB3.0 bus. In case the camera is connected to the slower USB2.0 Bus only the 13 MHz mode is available. At the slower 13 MHz the signal quality is slightly improved due to a reduced noise of the signal transmission.

Cooling

Status information, if camera cooling is active. The Axiocam 506 can be operated without active cooling. Cooling is deactivated if the USB2.0 connector of the camera is not supported to the PC or to a USB compatible power supply.

Readout Port

The Axiocam 506 uses a high performance CCD sensor with four readout ports. It can be adjusted to quadport, dualport, singleport and Auto mode. Maximum speed is reached by using all four ports and short exposure times. When exposure time gets larger than the readout time the benefit for using multiple ports is getting insignificant. By switching the readout mode to single port, the most homogenous signal quality can be reached as all data is sent through one single processing chain. In Auto mode, the number of used readout ports is selected automatically depending on the exposure time.

Readout Time

The valid camera readout (in ms) time is given in this status window which is defined by the number of used ports or by defining a sensor sub region window (ROI).

Temperature

The valid CCD sensor temperature is shown here. It is adjusted to 18 C°. It can not be changed. If a black reference is used it should be used at the same sensor temperature when it was created. If free air circulation for the camera housing is blocked, it may happen that the sensor temperature is increased and the dark current of the sensor may be higher than normal. If the camera is operated without cooling (USB 2.0 port of camera not connected) the sensor temperature is increased and dark current will be higher than normal. This should be considered when using the camera at longer exposure times.

13.3.1.2.9.2 Axiocam 712 / 705

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 90 CW**
- **Rotate 90 CCW**
- **Rotate 180**
- **Mirror at +45 Diagonal**
- **Mirror at -45 Diagonal**

Acquire section

Readout Speed (MHz)

Readout speed can be varied between 39 MHz and 13 MHz if the camera is operated on the USB3.0 bus. In case the camera is connected to the slower USB2.0 Bus only the 13 MHz mode is available. At the slower 13 MHz the signal quality is slightly improved due to a reduced noise of the signal transmission. In case of the 7 series Axiocam models, the readout clock speed is 37 MHz. This speed is fixed and can not changed.

Cooling

Status information, if camera cooling is active. The Axiocam models of the 5 and 7 series can be operated without active cooling. Cooling is deactivated if the USB2.0 connector of the camera is not supported to the PC or to a USB compatible power supply.

Readout Time (ms)

The valid camera readout time is given in this status window which is defined by the number of used ports or by defining a sensor sub region window (ROI).

Post Processing section

SW Subsampling

The software subsampling feature is used to reduce the image size, which helps to reduce the amount of the image data load in case of long time lapse or multi-dimensional images. As this is done by software processing, the full image is acquired and prepared before it is downsized by this function. Especially in high magnification objectives at lower NA values, the image resolution is optically limited. So this function can be used to minimize empty image information. This feature works with all Axiocam 3 series, 5 series, and 7 series models. In case of the 7 series models, the on chip sub sampling offers a speed benefit.

Temperature

The valid sensor temperature is shown here. It is adjusted to 18 C°. It can not be changed. If a black reference is used it should be used at the same sensor temperature when it was created. If free air circulation for the camera housing is blocked, it may happen that the sensor temperature is increased and the dark current of the sensor may be higher than normal. If the camera is operated without cooling (USB2.0 port of camera not connected) the sensor temperature is increased and dark current will be higher than normal. This should be considered when using the camera at longer exposure times.

13.3.1.3 Movie Recorder Tool

Here you can acquire image sequences in the form of videos using the camera's fastest burst mode.

Info

To play the acquired Movie, use the **Player** tab in the **Center Screen Area** (Only visible in **Show All** mode).

Parameter	Description
Start Movie	Starts the acquisition. The button changes into the **Pause** button. The animated **Stop** button appears in the window above the button.
Pause Movie	Pauses the acquisition. The button changes into the **Continue** button.
Continue Movie	Continues acquisition if it has been paused. The button changes into the **Pause** button.
Stop	Stops acquisition. Save the acquired movies either ▪ in the internal **CZI** format via **File** menu | **Save as...** ▪ as a series of individual images via **File** menu | **Export/Import** | **Export** ▪ or as an **AVI** file via **File** | **Export/Import** | **Movie Export**.

13.3.1.4 Live Panorama tool

With this tool you can acquire a panorama image exceeding the size of a single image.

Parameter	Description
Start Live Panorama	Starts the acquisition. The button disappears. The animated **Stop** button appears in the window above the button. For more information, see *Acquiring a Panorama Image Automatically [▸ 49]*.

13.3.1.5 Manual Extended Depth of Focus Tool

Parameter	Description
Z-Stack	If activated, an EDF image is acquired out of a Z-Stack image.
Quality	Here you can select the quality level that you want the function to work with.
Registration Method	Here you can select the method (or a combination of these) to be used to align the images.
- Translation	The neighboring sections of the Z-stack image are shifted in relation to each other in the X and Y direction.
- Rotation	The neighboring sections of the Z-stack image are rotated in relation to each other.
- Iso Scaling	The magnification is adjusted from section to section.
- Skew Scaling	The neighboring sections of the Z-stack image are corrected for skewness / shearing.
- Affine	The neighboring sections of the Z-stack image are shifted in the X and Y direction, rotated and the magnification is adjusted from section to section.
Interpolation	Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.
Mode	
- Timer	Acquires an EDF (Extended Depth of Focus) image automatically after the interval you have set.
- F12 Key	Acquires an EDF image when you press F12 key.

Parameter	Description
Interval	Only active, if you have clicked on the **Timer** button. Set interval (in sec.) here, after which the automatic acquisition begins.
Start	Starts the acquisition of an image series. Press the **Pause** button to pause acquisition. Press the **Continue** button, to continue acquisition. Press the **Stop** button to stop acquisition. The image with extended focus will be calculated from all single images.

13.3.2 Tools on Acquisition Tab

13.3.2.1 Imaging Setup Tool

Only visible, if the **Enable Imaging Setup** checkbox in the **Tools > Options > Acquisition > Acquisition Tab** is activated.

View and adjust the hardware parameters used for confocal (LSM) or camera (WF) experiments. All hardware parameters set to detect one or more specific signals simultaneously are defined as one track. Specific tracks can be combined for image acquisition which is then called a multitrack acquisition.

Info

Note that the **Microscope Control** tool on the **Locate** tab has a similar appearance and similar control elements but its function differs from the **Imaging Setup** tool described here.

If there is no track configured, you need to add a first track to the experiment by clicking on the drop down button. To see a list of the availalbe acquisition modes, click on the arrow on the right side of the button.

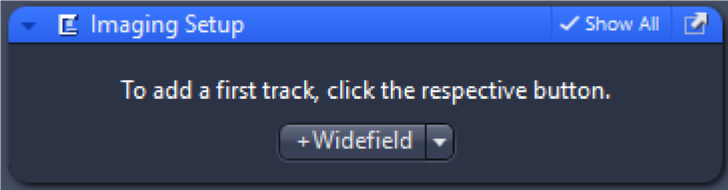


Fig. 58: Imaging Setup without tracks

If tracks are available each track will show as a button with the respective name (**Track1**, **Track2**, etc.) on the top of the tool. The active track is highlighted in blue color and the type of acquisition track is indicated below the button. Some tracks can be combined for image acquisition and run as a multitrack acquisition. All active tracks for acquisition are either blue (current active track and settings displayed below) or dark grey with white writing. Inactive tracks not used for acquisition are dark grey with grey writing. Tracks which cannot be combined for image acquisition with the current active track(s) are two-dimensional grey with grey writing. Switch between the tracks by clicking on the corresponding track button. Use the **Delete** button to delete tracks and access further options for editing via the **Options** button (Add, Delete, Duplicate, Rename).

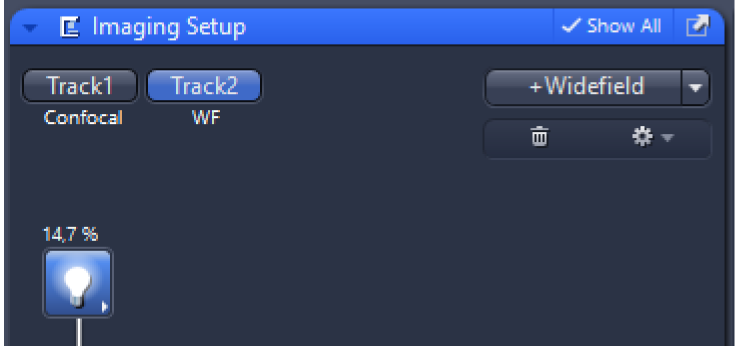


Fig. 59: Imaging Setup with tracks

Depending on what kind of track you have selected the **Imaging Setup** tool has a different appearance. For a detailed description of the specific parameters read the chapters *Imaging Setup (WF) [▸ 685]* and/or *Imaging Setup (LSM 980) [▸ 963]*.

Tracks of different kind are not always compatible with each other. It is e.g. not possible to combine camera and LSM acquisition into one single image. The Imaging Setup and Channels tool will automatically disable incompatible tracks upon creation of new tracks. To re-enable incompatible tracks, you need to deactivate first the active tracks. Once all incompatible tracks are disabled, the remaining tracks can be activated again.

13.3.2.1.1 Imaging Setup (WF)

By selecting a WF track you can see the graphical display of the acquisition light path with various icons. The arrangement of the icons represents the typical set-up of the microscope components configured on your system. For a description of the most common icons, read Reflected/Transmitted Light Path.

The associated hardware settings are shown above the icons and can be changed here. To change the relevant hardware settings, left-click on the icons. In the shortcut menus you will see numerous selection and setting options for adjusting your settings.

Info

Any change you make is automatically adopted and written to the corresponding hardware setting of the experiment. If you want to undo these changes, do not save the experiment. Instead, reload the experiment in the **Experiment Manager**.

If you change the hardware settings in this section, please bear the following points in mind:

- If the checkbox **Include in this setting** is activated the component is activated. Activated components are included into the hardware settings of the experiment and subsequently applied in the experiment. Activated components are highlighted in blue color.
- Components with a deactivated checkbox are not adopted into the hardware settings of the experiment and are not subsequently applied in the experiment. These components are displayed with a grayed-out icon.
- Components with a filled-in checkbox and a triangle underneath are only partially adopted into the hardware settings of the experiment and subsequently applied in the experiment. To show the sub-components, click on the triangle under the checkbox. To adopt the sub-components into the hardware settings of the experiment and subsequently apply them in the experiment, activate the relevant checkboxes for the sub-components.

13.3.2.1.2 Advanced Imaging Setup

Only visible, if the **Enable Advanced Imaging Setup** checkbox in the **Tools** menu | **Options** | **Acquisition** | **Acquisition Tab** | **Enable Imaging Setup** is activated.

There is a switch on top of the tool. By clicking on the button you can switch from the **Standard** to the **Advanced** Imaging Setup.

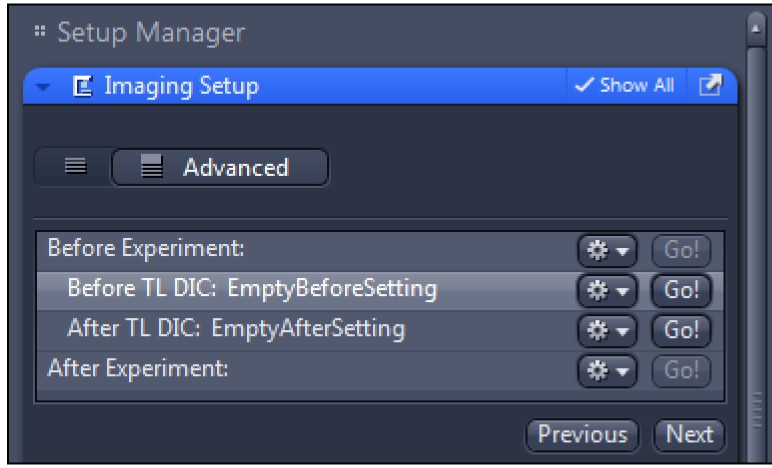


Fig. 60: Advanced Imaging Setup

The Advanced Imaging Setup offers additional options for controlling an experiment. The **Before/After Experiment** settings can be found right under the Standard/Advanced button.

Additionally the **Experiment Settings Pool** can be found at the bottom of the tool. We recommend that you talk to ZEISS Service staff or Imaging Specialists before you try to change settings here, as they should be changed only if you know what you are doing.

Before/After Experiment settings

Parameter	Description
Before/After Experiment	Shows the name of the hardware setting that will be applied immediately before or after the experiment.
Options	Opens the **Options** menu for the specific hardware setting.
Go!	Applies the selected hardware settings.
Previous/Next	The buttons allow you to navigate through the various hardware settings.

Experiment Settings Pool

Parameter	Description
Clear all unused hardware settings from experiment	By clicking on the **Clear** button you can delete all unused hardware settings from your experiment.
All available hardware settings in experiment	Here you can see all available hardware settings.

13.3.2.2 Acquisition Mode Tool

In the **Acquisition Mode** tool you can set the various acquisition parameters that you want to apply for the entire experiment.

Info

If you have created an experiment using the **Experiment Designer** tool, the settings in the **Acquisition Mode** tool only apply to the relevant experiment block and may differ in the next block.

In terms of content and appearance, the Acquisition mode tool is largely dependent on which imaging mode was chosen in the **Imaging Setup tool**, either LSM tracks or widefield channels.

If you have configured LSM tracks, including e.g. Airyscan or Lambda Tracks, please read the chapter *Parameters for LSM Imaging Modes [688]*.

If you have configured widefield channels, please read the chapter *Parameters for Widefield Mode [692]*.

13.3.2.2.1 Parameters for LSM Imaging Modes

Adjust scanning and acquisition parameters that you want to apply for the entire experiment.

In case the experiment is run as a multiblock experiment, the settings apply to one experiment block only.

Note that the available controls vary with the chosen track mode for LSM:

- LSM confocal
- Airyscan SR
- Airyscan MPLX
- NDD
- LSM Lambda
- LSM Online Fingerprinting

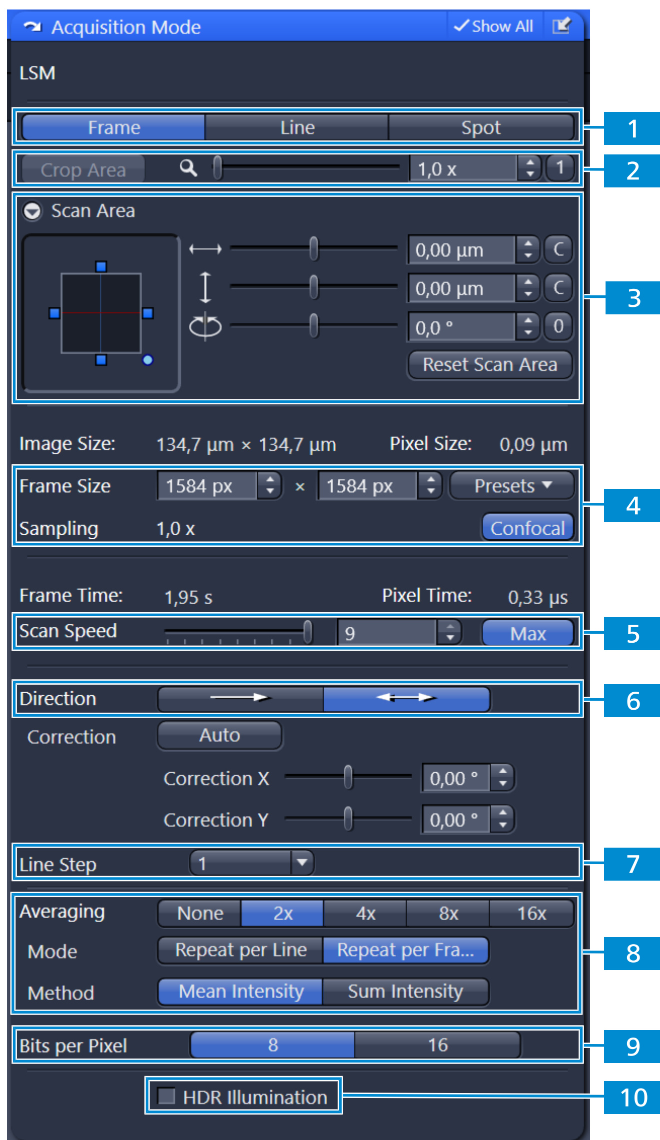


Fig. 61: Acquisition Mode tool

No.	Parameter	Description
1	**Scan Mode**	Select the Scan Mode.

No.	Parameter	Description
	- Frame	Activates the frame scan mode. If this mode is selected you will see the representation of the scanning frame in the **Scan Area** section.
	- Line	Activates the line scan mode. If this mode is selected you will see the representation of the scanning line in the **Scan Area** section. A line scan must be 128 pixels or more. Lower values are set back to 128 pixels. A line scan cannot be used to define a bleach region within a time series. Not available for **Airyscan** acquisition, as at least 32 lines are required to perform Airyscan processing.
	- Spot	Activates the spot scan mode. If this mode is selected (only available for LSM confocal mode) the scanner is stationary at a spot and the signal intensity is acquired from this one position. Note that one acquisition cycle / Snap consists of 1000 pixels each with the defined integration time. When acquiring a time series in spot scan mode, higher scan speeds will result in one or more dark pixels at the beginning of each acquisition cycle. Reduce the speed to avoid this behaviour. **Spot** scan cannot be combined with an acquisition region defined in the **Experiment Regions** tool.
2	**Crop Area**	Clicking the button projects a rectangular overlay into the center of the displayed image document representing the area of 2 x zoom. The overlay graphics can be adjusted to the sample region to be imaged in any rectangular rotated or non rotated from within the boundaries of the scan field. The subsequent image acquisition will then be confined to this area. Note that with a tile image the function will fail if the current stage position is not in the center position of the tile region because **Crop Area** is confined to the **Scan Area** and does not move the stage.
	- Zoom	Adjust the zoom level from **0.5x** (0.6 for LSM 980 on Axio Observer and Axio Imager; 0.7 for LSM 980 on Axio Examiner) - **40x** by using the **Zoom** slider. You can also enter a specific value in the input field. If clicking on the **1** button behind the input field the zoom level will be reset to default (1,0x) for confocal acquisition, 1.7 for Airyscan SR tracks on LSM 980; and 1.3x for Airyscan tracks using LSM 900.

No.	Parameter	Description
3	**Scan Area**	In this expandable section, you can adjust the position of the scan area. The outer frame corresponds to the field of view of the microscope. The inner frame represents the scan area. All changes of **Offset** and **Rotation** made in this section will be immediately applied to the scan area. Following functions are available:
	- Offset	Adjust the offset by using the **Left** / **Right** or **Up** / **Down** sliders. You can also enter a specific value in the input field. If clicking on the **C** button behind the input filed the offset position will be reset to center position. If you left click on the inner frame and hold down the mouse button you can move the scan area freely. The positions in the input fields will be adopted according to your adjustments.
	- Rotation	Adjust the rotation degree by using the **Rotation** slider. You can also enter a specific value in the input field. If clicking on the **O** button behind the input field the rotation degree will be reset to default position (zero degree).
	- Reset Scan Area	Resets all adjustment of **Rotation**, **Offset** and **Zoom** to the system defaults.
4	**Frame Size** / **Sampling**	Adjust the frame size (in pixel) of the displayed image by entering the desired value in the two input fields.
	- Presets button	By clicking on this button you can select from a list of default frame sizes (e.g. 128 x 128 or 512 x 512). We recommend to start with 512 x 512 px.
	- Confocal button	By clicking on this button the frame size (image resolution) will be set to an optimal value corresponding to the optical magnification (objective), the zoom factor and the wavelengths included in the experiment. This provides an image where no spatial information is lost and no empty data is generated as optimal sampling is achieved. The confocal value is calculated for the given objective and magnification settings matching a 1 fold sampling according to 1 time Nyquist. Rectangular image dimensions are preserved. When you press this button once, it will change its color to blue. This indicates that the optimal sampling will be maintained as you continue to change acquisition parameters like zoom, Laser lines or add more tracks. This active state of the button will be lost once you manually edit the frame size or click the button again.
	- SR button	Clicking this button (for Airyscan SR mode only) sets the sampling to 2x Nyquist for superresolution mode.

No.	Parameter	Description
5	**Scan Speed**	Set the scan speed by adjusting the slider from 1 (slow) to 19 (for LSM 900 the limit is 16) (very fast). The corresponding values for **FrameTime** and **Pixel Time** will be displayed above the slider. Please note that the available maximum scan speed depends on the selected **Frame Size** and zoom factor. Maximum Speed is available with a zoom factor of 13.2 (LSM 980) or 6.5x (LSM 900). By clicking on the **Max** button the maximal possible scan speed will be set automatically. When you click this button once, it will change its color to blue. This indicates that the system will always use the highest possible scan speed as you continue to change acquisition parameters like zoom or frame size. Click the button again to deactivate this permanently active state.
6	**Direction**	Following scanning directions can be selected:
	- Unidirectional	The laser scans in one direction only, then moves back with beam blanked and scans the next line.
	- Bi-directional	The laser also scans when moving backwards, i.e. the scan time is reduced by about a factor of two. Please note that the pixel shift between forward and backward movement (double image) resulting from bi-directional scanning must be corrected. To do that use the **Correction X** / **Correction Y** sliders. By clicking on the **Auto** button an automatic scan correction will be performed. For optimal results this correction should be repeated every time scan parameters like rotation, frame size, zoom or speed are changed.
7	**Line Step**	Only available if the **Frame** Scan Mode is selected. Select the desired line step size (from 1-10). According to your selection only every n-th line is scanned. The lines in between are interpolated. This fast scan mode is called Step Scan. This feature is not available for Airyscan acquisition.
8	**Averaging**	
	- Number	Select the number of images you want to average (2x - 16x).
	- Mode	Select the mode for averaging : ▪ **Repeat per Line**: The calculation of the average is based upon individual lines, which are acquired sequentially before moving on. By this, the data used for the averaging are is closely connected, but the acquisition of the full frame takes respectively longer.

No.	Parameter	Description
		▪ **Repeat per Frame**: The calculation of the average is based on individual full frames. Each frame is scanned quickly, but there is a higher delay between the individual data sets taken for the averaging.
	- Method	Select the method which will be used for averaging: ▪ **Mean Intensity**: Uses the mean average of all images ▪ **Sum Intensity**: Uses the sum of all images.
9	**Bits per Pixel**	Use the buttons to adjust the color bit depth to 8 Bit or 16 Bit (i.e. 256 or 65536 gray values).
10	**HDR Illumination**	This parameter is only available if you have licensed the **HDR Confocal Basic** module. If activated, a HDR effect will be applied to the image. This effect will boost weak structures without saturating bright areas in the image and enable an optimal representation of the morphology of weak and bright objects within the same image. To achieve this, the image will be scanned three times with increasing the excitation intensity. Areas in the image, that displayed overexposure will be excluded in the following scans in order to avoid photobleaching. It is recommended to use 16bit for the acquisition of HDR datasets. Note: HDR imaging is not possible when using an Experiment Region for Acquisition (see Experiment Regions Tool); whatever is activated first (HDR or Experiment Region for Acquisition) will then not allow to activate the other function. *HDR was developed based on ideas and a concept of O. Ronneberger and R. Nitschke (Albert-Ludwigs-University Freiburg, Department of Computer Science and Life Imaging Center at ZBSA).*

13.3.2.2.2 Parameters for Widefield Mode

13.3.2.2.2.1 Camera Section

In this section you can adopt camera settings from the active camera to your experiment and adjust basic camera settings.

Parameter	Description
Get Settings from Active Camera	
- Get	Applies the settings from the active camera to your experiment.
- Default	Resets the camera settings to factory default.

Parameter	Description
Binning	Here you can set the binning. Binning combines the information of neighboring camera pixels into a single larger pixel. For example, if the binning is set to 2 × 2, four pixels are combined to one. Increasing the binning means weaker signals can be detected for a given exposure time. For CCD cameras, binning increases sensitivity by improving the signal-to-noise ratio, with resolution being decreased by the same factor. In the case of CMOS cameras, only the signal intensity is increased and the pixel count and resolution gets reduced correspondingly.
Binning-independent Brightness	Only visible when the **Show all** mode is activated. Because **Binning** generally increases signal intensity, the brightness of the image normally also increases correspondingly. By activating this checkbox, the brightness level is automatically fixed (depending on the camera, either through exposure time adjustment or averaging), no matter the binning setting.
IP Quality	Here you can select the color interpolation quality (**IP Quality**) for the acquired image. Please note that this function does not apply to **Live** mode. **Fast**: color interpolation for optimum speed (shorter computation). **High**: color interpolation for optimum quality (less artifacts). This mode is only effective with binning factor 1.
Subsampling	Here you can reduce the amount of data acquired to achieve faster frame rates. By subsampling 2x2, the effective pixel pitch is increased by sampling only every other pixel, thus reducing the overall data size of your image. The modern CMOS sensors in the Axiocam 705 and 712 offer a on-chip subsampling mode. This subsampling mode enables a dramatic increase in frame rate especially for time series acquisition (at short exposure times) at an full field of view of the camera. In addition, the amount of produced image data is decreased. As this is done by skipping every second pixel in x and y direction, the optical resolution is decreased accordingly. Especially in high magnification objectives at lower NA values, the image resolution is optically limited. So this function can be used to minimize empty image information.
Resolution	Displays and selects the camera resolution, e.g. 1024 x 1024 px.

13.3.2.2.2.2 Acquisition ROI Section

In this section you can define a **Region Of Interest (ROI)** on the camera sensor which will be used for acquisition. A smaller ROI can increase the acquisition speed.

The region of interest is indicated by a blue frame in the preview window and can be moved and resized freely. The preview window always shows the entire camera sensor area which can be acquired.

The **Pixel Size** shown below the preview window indicates the size in µm to which a pixel corresponds. This depends on the camera sensor properties and on the binning.

Parameter	Description
Maximize	Selects the entire available image sensor area as the region of interest.
Center	Positions the region of interest precisely at the center of the image.
Size	Sets the width and height of the region of interest in pixels.
Offset	Specifies the position of the top left corner of the **Acquisition ROI** (blue frame) with respect to the top left corner of the preview window.
Refresh Overview	Acquires and displays an image in the preview window with the current ROI settings. This has no effect on the image in the **Center Screen Area**.
Crop	Allows you to specify a ROI (Region of Interest) in a snapped image. The "cropped" area is used as ROI for the next image acquisition. If no image is available in the **Center Screen Area** the button is deactivated.

13.3.2.2.2.3 Fast Acquisition Section

In this section you can set 3 different modes for acquisition.

Parameter	Description
Interactive	Using this mode you can intervene manually at certain points during acquisition. The acquisition is comparatively slow.
Compromise	This mode is activated automatically if only individual hardware components, but not the whole system, are compatible with the Triggered mode for acquiring an experiment.
Triggered	Fast acquisition via the hardware.
- Validate	To establish whether the system is able to perform an experiment in **Triggered** mode, click on the **Validate** button. The validation result is displayed in the info box below the button bar.

13.3.2.2.2.4 Post Processing Section

In this section on the **Acquisition** Tab in the **Acquisition Mode** tool you can apply basic image processing functions while acquiring the image. This can be helpful if certain image processing steps are necessary for any acquired image and saves image processing work later in a job. Depending on the camera model, different parameters can be available.

Parameter	Description
Black Reference	Influences the live image and each image acquired. For the black reference to work, you first need to acquire a reference image. Define a corresponding reference image using the **Define** button. **Activated:** Applies the measured black reference to the image. **Deactivated:** The measured black reference is not used. The reference image is retained. If longer exposure times are used (from exposure times of approx. >5s, depending on the camera), individual bright pixels may become visible with CCD or CMOS sensors. With the help of the black reference these effects are measured and corrected in accordance with the exposure time employed. It is recommended that you repeat this measurement at certain intervals. This correction is recommended in particular for applications that involve long exposure times, i.e. for which very little light is used (live cell imaging, fluorescence images). The availability of a black reference for the selected camera can be checked on the menu **Extras** | **Calibration Management**.
- Define	Automatically defines the black reference. The measurement lasts for several seconds. The **Black Reference** checkbox is then activated automatically.

Info

For the measurement the camera must see a completely dark image. The light path to the camera must therefore be blocked. Set the eyepiece switch to 100% eyepiece and close the reflected light/transmitted light shutter. To define the black reference, click on the **Define** button.

Parameter	Description
Enable Noise Filter	**Activated:** Filters the noise in the acquired image according to the adjusted threshold. Affects acquired images only. The live image does not change. Deals with sporadic bright events in single pixels on CMOS sensors, like blinking pixels in dark images or spurious cosmic ray events. This feature is most useful for situations with high gain settings for acquisition of very dim signals with CMOS or EMCCD cameras. In combination with a correct black reference an absolutely flat signal background can be produced.

Parameter	Description
- Threshold	The higher the value, the greater the tolerance for noise. The lower the value, the stronger the noise reduction. The noise filter reduces the extent to which individual pixels deviate from the average value of their nearest neighbors. The **Threshold** corresponds to a tolerance value. If the deviation of the middle pixel value from the average value of the pixels immediately surrounding it exceeds the tolerance value (i.e. it is interpreted as noise), it is replaced by the average value. This technique reduces the noise of individual pixels that are produced, in particular with EMCCD cameras and CMOS cameras. The selected technique prevents any changes being made to object edges, as in most cases these are larger than individual pixels. This filter is also suitable for removing individual "hot pixels" from an image without having to acquire a reference image in advance.
Enable Unsharp Mask	Enhances contrasts at fine structures and edges. Thus, the resulting image appears clearer and enriched in detail.
- Strength	Controls the amount of contrast enhancement applied to fine structures and edges. The higher the strength, the darker or lighter the resulting edges, compared to the original image.
- Radius	Determines the size of detail to be enhanced. A small radius enhances smaller details. The radius also affects the appearance of enhanced edges. A large radius leads to a visible halo along enhanced edges. The larger the radius, the broader the halo.
- Color Mode	Determines the calculation method, which affects the appearance of the output image. ▪ **RGB**: – The **Unsharp Mask** filter calculates the sharpness for each color channel individually. – The color saturation and the color of structures may be changed and color noise may occur. ▪ **Luminance**: – The **Unsharp Mask** filter calculates the sharpness based on the luminance signal computed from the RGB channels. – This mode avoids possible color noise or shift in color saturation, which could be induced by certain image textures.
- Auto Contrast	**Activated:** Enables you to adjust the **Contrast Tolerance** (0-20). **Auto Contrast** only works in RGB color mode.

Parameter	Description
- Contrast Tolerance	Increasing the contrast during unsharp masking is achieved by broadening the distribution of intensities. This corresponds to a spread of the image histogram. Controls how much the intensity distribution is spread and thus how strong the contrast is increased. ▪ **Contrast Tolerance = 0** : No spread of intensities, no increase of contrast ▪ **Contrast Tolerance = 20**: Maximum spread of intensities, maximum increase of contrast
- Clip To Valid Bits	**Activated:** Composes the processed image of the same colors as the original image (i.e. the value range of the output image is adjusted to the color range of the input image). **Deactivated:** Colors not present in the original image may appear in the processed image.

13.3.2.2.2.5 Mode Section

In this section you can adjust how the software retrieves the camera sensor data.

Parameter	Description
Color Mode	This parameter is available for color cameras only.
– RGB	Transmits the image data of a color camera unchanged. This corresponds to the standard operating mode of a color camera.
– B/W	Treats the image data of the color channels as grayscale. The data of related color channels are averaged. The saturation of the camera appears reduced as a result. This process does not change the spectral properties of a color camera. The image information of the color sensor still undergoes color interpolation. An infrared filter also restricts the spectral sensitivity of the color camera compared to the spectral sensitivity of a genuine black and white camera.
Live Speed	Here you can select the live image update speed (For CCD based Axiocam models = slow, medium, fast). Enables you to focus or to find regions of interest on a sample quickly. A high live image update speed reduces the exposure time of the live image, even at longer exposure times used for image acquisition. To achieve a similar impression of image brightness, however, the image data supplied must be adjusted digitally, which may generate a certain amount of noise or reduce the resolution of the live image. For CMOS sensor based camera model (e.g. 712 / 705) High Res, Fast, Low Light).
– Fast	Represents the image optimized and requires a shorter computation time.
– High	Represents the image without artifacts and with a higher image quality. This mode is only effective with binning factor 1.

Parameter	Description
Bit Depth	The **Bit Depth** function enables the reduction of the delivered camera bit depth. It is translating the 14 bit based camera data values into a smaller value of 12 bit or 8 bit. This has an visible effect of the number range in the image histogram, the dynamic range and the corresponding image file size in an uncompressed CZI image. As default, the Axiocam models deliver 14 bit per pixel (Axiocam 503/ 506/ 512/ 702) and 12 bit/pixel in case of the Axiocam 305. 14 bit and 12 bit per pixel values need to be stored in two bytes in an CZI image file. In case of an translation to 8 bit per pixel, only one byte is needed. Therefore it is possible to reduce the produced image file size by a factor of two. This is especially beneficial, if large amount of image data is acquired. By reducing the native camera bit depth to smaller values, the available number range for the digital image signal is decreased. Therefore, the available amount of resolvable intensities in one image scene is decreased accordingly.
– LUT min/LUT max (only available for 8 bit mode)	LUT stands for Look Up Table. It describes the translation method for quickly translating digital numbers in a different range. If the full swing of the input signal is not used, the reduction of bit depth can be adopted accordingly by the translation starting point "Lut min" and the translation end point "Lut max". If the used intensity range equals just an 8 bit value range, no information is lost and the unused bits can be excluded from being stored by an suitably adjusted translation table. Available range is 0 to 1. The value 0 equals 0% of the input range. The number 1 equals 100% of the input range.
– Gamma (only available for 8 bit mode)	The 14 bit to 8 bit translation is linear by default, which equals a Gamma value of 1. By assigning values larger or smaller then 1, the translation becomes non linear. Values <1 selectively reduce dim signal intensities. Values >1 selectively amplify dim signal intensities.

13.3.2.2.2.6 Model Specific Section

Only visible if the **Show All** mode is activated.

To show the section in full, click on the **arrow** button .

In this section you see additional, model-specific camera settings depending on which camera you use on your system.

Reset button

Resets all entries to the original values.

13.3.2.2.2.6.1 Axiocam 105

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 180**

Acquire section

Gain Boost checkbox
If activated, the image signal is amplified so that the image becomes brighter. The gain factor is 1.7x. This factor is in addition to the standard Gain control.

13.3.2.2.2.6.2 Axiocam 712 / 705

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 90 CW**
- **Rotate 90 CCW**
- **Rotate 180**
- **Mirror at +45 Diagonal**
- **Mirror at -45 Diagonal**

Acquire section

Readout Speed (MHz)

Readout speed can be varied between 39 MHz and 13 MHz if the camera is operated on the USB3.0 bus. In case the camera is connected to the slower USB2.0 Bus only the 13 MHz mode is available. At the slower 13 MHz the signal quality is slightly improved due to a reduced noise of the signal transmission. In case of the 7 series Axiocam models, the readout clock speed is 37 MHz. This speed is fixed and can not changed.

Cooling

Status information, if camera cooling is active. The Axiocam models of the 5 and 7 series can be operated without active cooling. Cooling is deactivated if the USB2.0 connector of the camera is not supported to the PC or to a USB compatible power supply.

Readout Time (ms)

The valid camera readout time is given in this status window which is defined by the number of used ports or by defining a sensor sub region window (ROI).

Post Processing section

SW Subsampling

The software subsampling feature is used to reduce the image size, which helps to reduce the amount of the image data load in case of long time lapse or multi-dimensional images. As this is done by software processing, the full image is acquired and prepared before it is downsized by this function. Especially in high magnification objectives at lower NA values, the image resolution is optically limited. So this function can be used to minimize empty image information. This feature works with all Axiocam 3 series, 5 series, and 7 series models. In case of the 7 series models, the on chip sub sampling offers a speed benefit.

Temperature

The valid sensor temperature is shown here. It is adjusted to 18 C°. It can not be changed. If a black reference is used it should be used at the same sensor temperature when it was created. If free air circulation for the camera housing is blocked, it may happen that the sensor temperature is increased and the dark current of the sensor may be higher than normal. If the camera is operated without cooling (USB2.0 port of camera not connected) the sensor temperature is increased and dark current will be higher than normal. This should be considered when using the camera at longer exposure times.

13.3.2.2.2.6.3 AxioCam ERc5s

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Sharpness

Using this function you can increase the impression of sharpness in an image.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 180**

13.3.2.2.2.6.4 AxioCam ICc5

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 90 CW**
- **Rotate 90 CCW**
- **Rotate 180**
- **Mirror at +45 Diagonal**
- **Mirror at -45 Diagonal**

13.3.2.2.2.6.5 AxioCam HRc

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 90 CW**
- **Rotate 90 CCW**
- **Rotate 180**
- **Mirror at +45 Diagonal**
- **Mirror at -45 Diagonal**

Readout Speed (MHz)

The **High Speed** mode activates the faster 24 MHz mode, for which the digitization accuracy is set to 12 bits per pixel. This mode offers advantages if sufficient light is available and situations need to be acquired quickly: fast time series or tile images.

In **High Accuracy** mode the readout speed is 12 Mhz and the digitization accuracy 14 bits per pixel. This mode offers advantages if very little light is available and you want the camera to acquire very weak signals just above the camera's noise level.

13.3.2.2.2.6.6 AxioCam MRm

Camera Identifier

A clear identifier for the currently active camera is displayed here. It consists of the product name and part of the serial number. This is helpful if you want to identify the camera in question, e.g. when working with a dual camera system.

Orientation dropdown list

Here you can select the orientation of the camera image. This allows you to adjust the camera image to the properties of the different camera ports. The following selection options are available:

- **Original**
- **Flip Horizontally**
- **Flip Vertically**
- **Rotate 90 CW**
- **Rotate 90 CCW**
- **Rotate 180**
- **Mirror at +45 Diagonal**
- **Mirror at -45 Diagonal**

13.3.2.3 ApoTome Mode Tool

Parameter	Description
Enable ApoTome	**Activated:** Uses the ApoTome for acquisition and experiments.
Phase Images	Select here the number of phase images per optical sectioning. 5 phase images are the default value.
Live Mode	Here you set the display of the **Live Mode**. Default value is **Grid Visible**.

13.3.2.4 Channels Tool

Info

The basic **Tiles** tool is only visible if you have a motorized stage configured with your microscope. The **Tiles Advanced Setup** and many other functions are only available if you own the **Tiles & Positions** module and when it is activated in the **Modules Manager**. Additionally, you must activate the corresponding checkbox on the **Acquisition** tab in the **Experiment Manager**. This tool is part of the basic license for LSM.

In the **Channels** tool you can configure channels for Widefield acquisition. The tool offers you the option of entering the hardware settings for acquisition manually or performing the configuration automatically. Its is not recommenced for LSM channels as you still need to open up **Imaging Setup** or use **Smart Setup** to chose specific channels and adapt the spectral range for detection.

If there is no channel available, you will be asked to add a **LSM** (various modes) or a **WF** channel to the experiment. Therefore click on the corresponding button.

Fig. 62: Channels Tool (without channels)

The following tool appearance is only visible if you have added one or more channels in Widefield mode:

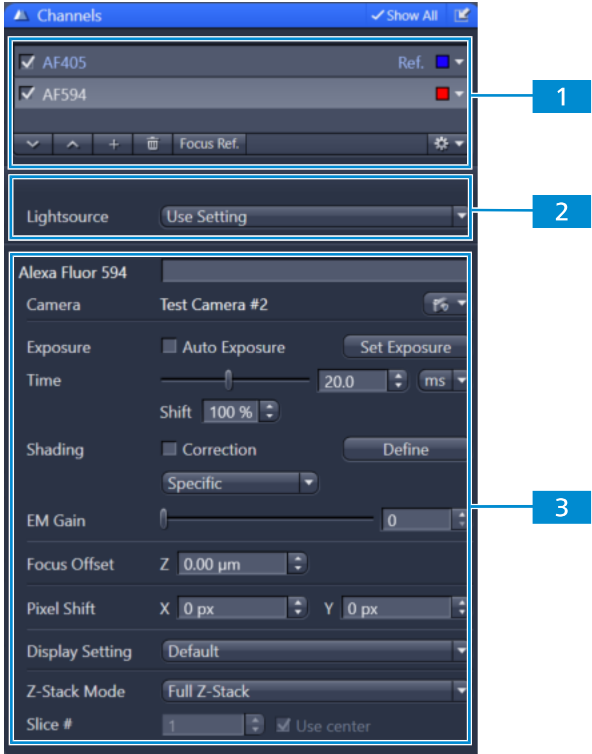


Fig. 63: Channels tool

1 Channels list
Displays the selected channels of your experiment. For more information, see *Channels list [▸ 704]*.

2 Light Source section
Displays settings for the light source or the lasers (LSM). For more information, see *Light Source section (WF) [▸ 706]* or *Lasers section [▸ 708]*.

3 Channel settings
Displays settings for your channels. For more information, see *Channel Settings (WF) [▸ 706] / Channel Settings (LSM) [▸ 710]*.

13.3.2.4.1 Channels list

Please bear the following points in mind for the channels list:

- The selected channel is highlighted by a light gray bar.
- The reference channel for the auto focus is highlighted by a blue font color and marked with **Ref.**.
- The preview color (LUT) for the channel is shown on the right side of the list. To change the preview color for the channel, click on the colored rectangle with the arrow icon and select an alternative color from the shortcut menu. The preview color is also shown in the sections for channel-specific hardware settings as a thin line on the left side of the list.

Info

If you select a dye or contrast technique in the **Add Dye or Contrasting Method** dialog, a suggestion for the hardware settings for the acquisition of this channel is made automatically. If no suggestion can be made, a channel without hardware settings is added. You will then see a corresponding indication in the status area of the program interface.

List control elements

Parameter	Description
Down	Navigates one row down.
Up	Navigates one row up.
Add	Opens the *Add Dye or Contrasting Method Dialog [▸ 809]*.
Delete	Deletes the selected channel.
Focus Ref.	Sets the selected channel as reference channel for focus actions or stitching during acquisition.
Options	Opens the *Options [▸ 705]* shortcut menu.

Laser Range section

Parameter	Description
High Intensity Laser Range	**Activated:** Uses a high intensity laser range , where you can adjust the lasers between 0.2 and 100 % of their power. This is especially relevant for bleaching experiments. The setting affects all tracks. While switching, the system is trying to keep the intensities at a similar level. If the currently selected intensity is outside of the overlapping range (0.2 % to 3.5-5 %) the closest possible value is used.

Parameter	Description
	Note: This section is only available with LSM 900. The LSM is working by default in a laser power range between 0.01 % and 3.5-5 % of the available laser intensity. The available minimum and maximum in the default range is depending on the laser.

13.3.2.4.1.1 Channels Tool Options Menu

Parameter	Description
Add New...	If **WF** channels are added this function opens the *Add Dye or Contrasting Method Dialog [▸ 809]* to add more WF tracks. If **LSM** channels were added this function will add a new LSM track.
Duplicate	Creates a new track with the same settings and dye as the currently selected track.
Rename	Assigns a new name to the channel of the currently selected track. To change the name of the track, you can directly click into the respective field of the channels list.
Reset Color	Resets the color of the selected track(s) to default.
Select All	Selects all tracks of the list.
Delete	Deletes the selected track.
Delete All	Deletes all existing tracks.
Set as Reference Channel	Defines the selected channel as the reference channel for focusing actions. Note that, you can also set Channels of inactive Tracks as Reference Channels. The Autofocus will in this case be performed on this channel, but the Channel or Track will not be part of the resulting image document. Using this approach, e.g. Camera tracks may be used for fast focusing within a Confocal experiment, while the acquisition of Camera and Confocal into one image is not possible. **Note:** Do not use a Lamdba or Online Fingerprinting track as reference track for SW Autofocus as the focus search will be extremely slow.
Compare...	Opens the **Compare channels** dialog, where the active channels are displayed horizontal so you can easily compare and adjust key parameters of the active tracks.

13.3.2.4.2 Channel-specific settings (WF)

The settings always relate to the channel you have selected in the **Channels** list.

To show the settings for all channels, click on the button | **Select All** in the **Channels** list.

13.3.2.4.2.1 Light Source section (WF)

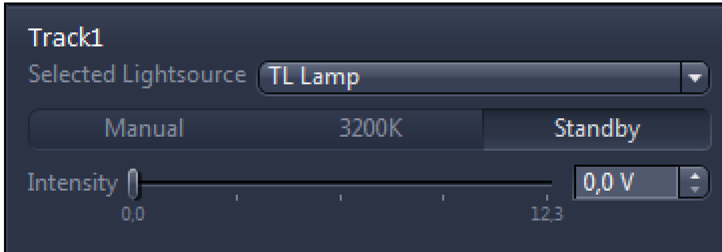


Fig. 64: Light Source Section

In this section you can select the available light sources from the **Selected Lightsource** drop-down list and adjust the corresponding settings. You can adjust the parameters of the light sources without having to save these in the hardware settings. You can therefore adjust the intensity of the laser lines or LEDs, for example, immediately before starting an acquisition.

If your system is equipped with a TIRF slider, the TIRF angle and type of illumination can also be set here.

If you select the **Use Setting** entry, the settings for the light sources disappear. The light source parameters from the hardware settings are used instead for the acquisition of the channel.

13.3.2.4.2.2 Channel Settings (WF)

Parameter	Description
Dye name	In the input field after the selected dye you can enter an additional name.
Camera	Select the desired camera for the channel from the dropdown list.
Time	Adjust the exposure time for the camera using the slider or spin box/ input field. Select the unit of time from the dropdown list at the right of the spin box/input field.
Shift	Only visible if the **Show All** mode is activated. Here you can enter the range of the camera's dynamic range that is utilized.
Auto Exposure	**Activated:** Automatically determines the camera's exposure time for the selected channel. The value set manually is ignored.
Set Exposure	Starts an exposure time measurement for the channel. After the measurement the value is adopted as the exposure time setting.
Shading Correction	**Activated:** Uses the calculated shading correction for this channel. To learn more about shading correction read the chapter, *Post Processing Section [▸ 673]*.
- Define	Automatically calculates the shading correction.
- Specific	Performs channel-specific shading correction.
- Global	Performs an objective specific shading correction. This is the default method for shading correction.
EM Gain	Only visible for EMCCD camera models. Sets the EM gain value.

Parameter	Description
Focus Offset	Only visible if the **Show All** mode is activated. Here you can enter the focus offset from the channel to the Z-position of the reference channel or to the current position. The **Lock** icon shows that this setting will be synchronized between all channels of this track.
Pixel Shift	Only visible if the **Show All** mode is activated. Here you can define the pixel shift in **X** and **Y**. The defined pixel shifts are applied to images collected with Snap, contentious or in an experiment.
Display	Only visible if the **Show All** mode is activated. Here you can select an existing predefined display setting to be automatically applied to this channel after acquisition. Please note, that this does not apply for Live acquisition. There always the last used setting will be applied.

Parameter	Description
Z-Stack Mode	Only visible if the **Z-Stack** checkbox is activated in the **Experiment Manager** and while **Show All** mode is active.
- Full Z-Stack	Acquires the Z-stack as defined in the **Z-Stack** tool.
- Single slice only	Acquires a single slice of the Z-stack only. Select the single slice in the input box under the list. If the **Use center** checkbox is activated, the center focus plane will be used for acquisition.
- Single slice, rest black	Acquires an image of a single slice of the Z-stack only. All other Z-slices of the stack are filled with black images. Select the single slice in the input box under the list. If the **Use center** checkbox is activated, the center focus plane will be used for acquisition.
- Fill with single slice	Acquires an image of a single slice of the Z-stack only and fills all other Z-slices with this slice. Select the single slice in the input box under the list. If the **Use center** checkbox is activated, the center focus plane will be used for acquisition.

13.3.2.4.3 Channel-specific settings (LSM)

The settings always relate to the channel you have selected in the **Channels** list.

To show the settings for all channels, click on the button | **Select All** in the **Channels** list.

If you are using FCS for LSM 980, see the chapter *Channels Tool - Measurement Settings [920]* for specific information.

13.3.2.4.3.1 Lasers section

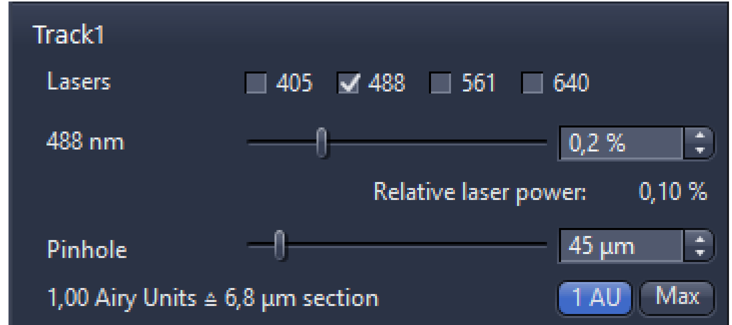


Fig. 65: Lasers Section

Parameter	Description
Laser	Select the lasers lines needed for sample illumination for the current track. Activate the required lasers by activating the corresponding checkbox. The laser lines along with sliders will appear. Set the required attenuation (%) using the sliders, the arrows, or typing a number into the input field. In case the laser is not yet ready for operation (see alsoLaser tool), the checkbox and text will be indicated in red. For tunable multiphoton lasers an editing field is displayed. Write the desired wavelength into the editing box to tune the laser to this wavelength. For multiple tracks the actual tuning is immediately done for the first active track. For subsequent tracks the tuning happens whenever the previous tracks are deactivated or deleted or during the actual experiment. In case a tunable and a fixed line are available, the tuning range has a gap which is defined by the filter combining the tunable and fixed line to get both beams onto the same optical path.
Relative laser power	Displays the relative percentage of the laser light that is applied to the sample. For more information, see *Display of relative laser power for different magnifications [▸ 709]*.
Pinhole	Adjusts the diameter of the pinhole. The diameter is specified in micrometer. The text below translates this diameter to Airy Units and section thickness which is grasped by the system for the configured detection range and excitation wavelength. The Airy Unit size and, derived from this, the section thickness of the optical slice is determined by the emission detection range. Diameter Pinhole = 1.22 x (detection wavelength / numerical aperture). For the calculation of the detection wavelength the center of the emission range is taken. Laser lines set within the detection range reduce the detection range (lower border determined by the laser line) and shift the center point accordingly. Without any defined detection range (i.e. no emission filter) the system takes the center of the potential detection range defined by the hardware parameters of the detector. Within one track the lowest detection range is taken for the calculation. When increasing the pinhole diameter an information mark might appear. It signals when at the given optical parameters the resolution in Z is no longer optimal. The tool tip showing with the information mark informs about what is the best step size for Z stacks to not loose resolution. For LSM confocal tracks using NLO lasers for excitation the

Parameter	Description
	section thickness is calculated based on a formula leaving out the settings of the pinhole, Hence no Airy Unit value is displayed. These tracks should always be setup with a completely open pinhole. The control is not available for Airyscan and NDD tracks. For Airyscan tracks the physical pinhole is automatically opened and set to the optimal diameter. NDD tracks do not have a pinhole.
- 1 AU	Sets the pinhole diameter to a value that is corresponding to 1 Airy unit for the configured detection wavelength, excitation wavelength(s) and objective. When you click this button once, it will change its color to blue. This indicates that the system will always use a pinhole diameter corresponding to 1 AU as you continue to change acquisition parameters like excitation or emission wavelengths. Click the button again to deactivate this permanently active state.
- Max	Opens the pinhole to its maximum diameter. This can be useful to find the focal plane and is the recommended setting when using a multiphoton laser for excitation.

13.3.2.4.3.1.1 Display of relative laser power for different magnifications

When using certain objective/ magnification changer combinations, only a portion of the laser light is applied to the sample due to the optical design of the system (see the table for applicable laser power below). This information is displayed as relative laser power (for LSM tracks) and relative laser power in confocal mode (for Airyscan MPLX tracks) in the **Channels** tool. The value is based on the currently used hardware settings (defined in the Right Tool Area) and does not reflect the before/after settings in experiments and experiment blocks.

The laser power is calculated in the following way:

- For confocal tracks: Relative laser power (%) = applicable laser power depending on objective/ magnification changer * adjusted laser power.
- For Airyscan tracks: Relative laser power in confocal mode (%) = applicable laser power depending on objective/magnification changer * equivalent laser power in confocal mode.

Applicable Laser Power (relative)

	5x/0,35	20x/0,7	20x/0,95	50x/1,2
0,5x	100%	49%	100%	92%
1x	100%	49%	64%	23%
2x	49%	12%	23%	6%

13.3.2.4.3.2 Channel Settings (LSM)

Parameter	Description
Master Gain	Slider and editing box to control the voltage of the PMTs. Increasing the gain of the PMT corresponds to a higher voltage of the detector. The image becomes brighter and you may be able to reduce the laser power. At higher voltage, the noise level in the image increases as the dark noise of the detector becomes visible in the images predominantly as single bright pixels. The optimum between gain and noise depends on your experimental requirements and on your sample. The maximum available voltage depends on the type of the detector and is 1200V for multialkali PMTs, 900V for GaAsP PMTs and 1000V for Airyscan. GaAsP PMTs and Airyscan have a minimum voltage of 500V.
Display	Select an existing predefined display setting to be automatically applied to this channel after acquisition. Please note, that this does not apply for Live acquisition. There always the last used setting will be applied.
Mode	For specific GaAsP channels and one cooled Multialkaline PMT of the LSM 980, the detectors can be run in photon counting mode. This optional mode is activated by clicking the button Photon Counting. In photon counting mode no Digital Offset and Digital Gain slider is available. Master Gain is set to a calibrated value to have optimal settings for photon counting. Offset must not be changed for this mode either. In photon counting mode an artificial maximum of detectable photons is set. For all GaAsP detectors and the side PMT (Ch2) this value is defined by CRmax/digital gain (CRmax = maximal count rate) defined by the software, which is 4 MHz for the BiG.2 and Ch2 and 2 MHz for Chs (32 channel GaAsP array). Higher count rates are regarded as saturation. Note that too much light on the detectors (higher count rates for some ms) leads to detector shut down to avoid damage. Note that images acquired in photon counting mode are displayed in their original count rate per pixel and hence will appear fairly dark compared to intensity mode images. Adjust the display to improve the signal visibility.

The following functions are only visible if the **Show All** mode is activated:

Parameter	Description
Digital Offset	Digital Offset allows to change the background of an image. It is calibrated such that the minimum grey values are set to zero. In case image information with zero as grey level value is not accepted increase the offset until no zero value pixel is present in the image. You can check this with the histogram function or the range indicator LUT. This control element is not available for Airyscan SR tracks.

Parameter	Description
Digital Gain	Digital Gain increases the amplification of the signal on the digital level. It is set to a value of 1. This value corresponds to 3 times the minimal possible value. This amplification provides a certain buffer for a possible detector overload. When decreasing the Digital gain to its minimum value of 0,3 an overload of the detector by increasing the master gain (or laser power) is reached earlier. Detectors will downregulate the voltage or shut down completely when partially or completely overloaded. Increase the digital gain and downregulate the master gain when very bright samples are imaged to avoid detector overload.

Parameter	Description
Z-Stack Mode	Only visible if the **Z-Stack** checkbox is activated in the **Experiment Manager** and while **Show All** mode is active.
- Full Z-Stack	Acquires the Z-stack as defined in the **Z-Stack** tool.
- Single slice only	Acquires a single slice of the Z-stack only. Select the single slice in the input box under the list. If the **Use center** checkbox is activated, the center focus plane will be used for acquisition.
- Single slice, rest black	Acquires an image of a single slice of the Z-stack only. All other Z-slices of the stack are filled with black images. Select the single slice in the input box under the list. If the **Use center** checkbox is activated, the center focus plane will be used for acquisition.
- Fill with single slice	Acquires an image of a single slice of the Z-stack only and fills all other Z-slices with this slice. Select the single slice in the input box under the list. If the **Use center** checkbox is activated, the center focus plane will be used for acquisition.

13.3.2.5 Focus Strategy Tool

Here you can select the focus strategy that you want to apply. The strategies that are available depend on the dimensions selected (Z-stack, time series, tiles), the hardware devices present (e.g. Definite Focus) and software licenses (e.g. **Software Autofocus** module).

In general focus strategies determine and/or update a Reference Z-Position, which in most cases is used directly for acquisition.

Info

The software automatically selects the most appropriate focus strategy e.g. **Use Z Values/ Focus Surface defined in Tiles Setup** when you activate the tiles dimension and no focus strategy i.e. other than **None** has been selected by you previously.

Exceptions

- When z-stacks are acquired, the center of the z-stack determines the Reference Z-Position.
- Defined offsets for channels and z-stacks shift acquisition in relation to the Reference Z-Position.
- If two focusing methods are combined, the Reference Z-Position of the first method is used as the starting point for the subsequent method.

Parameter	Description
Focus Strategy Wizard	
– Optimize this focus strategy	Opens the *Focus Strategy Wizard [▸ 716]* to optimize this focus strategy used for your tiles experiment.
Focus Strategy drop-down list	Depending on your system, experiment configuration and licensed modules, the following strategies are available:
– None	No focus strategy has been selected. This is the default setting for all experiments that do not include a tiles dimension. In this case the software automatically selects the **Use Z Values/ Focus Surface defined in Tiles Setup** strategy. The current z-position at the time the experiment is started is set as the Reference z-Position and remains unchanged during the experiment. **Exception:** By default, Z-stacks are acquired at the fixed Reference Z-Position that has been defined as the center in the Z-Stack tool. You can change this setting in the **Z-Stack Acquisition** section of the **Focus Strategy** tool.
– Absolute Fixed Z Position	Only available if the **Tiles & Positions** module is not licensed. A focus strategy that makes use of the z values defined for tiles and positions when using a motorized stage.
– Software Autofocus	Only available if you have licensed the **Software Autofocus** module. Note that any focus surface / z-values defined in the tiles setup are ignored if this strategy is selected. If you want to use the values setup in the **Tiles** tool select the strategy **Use Z Values/ Focus Surface defined in Tiles Setup**. The focus position is determined via the sharpness calculation or intensity calculation of a series of images (Z-stack) and set as the Reference Z-Position. The settings are configured in the **Software Autofocus** tool.
– Definite Focus	Only available if your microscope system has attached a **Definite Focus** device. Note that any focus surface / z-values defined in the tiles setup are ignored if this strategy is selected. If you want to use the values setup in the **Tiles** tool select the strategy **Use Z Values/ Focus Surface defined in Tiles Setup**. Definite Focus attempts to maintain a certain distance to the cover glass of the sample in order to compensate for mechanical and thermal movements. The Definite Focus is initialized at the start of the experiment by setting the current distance as the reference distance. You will be requested to define this value at the start of the experiment. When the focus is stabilized during the experiment, the current distance is adjusted to the reference distance. This is achieved by moving the focus drive accordingly. The new z-position resulting from this is used as the Reference Z-Position for acquisition. The repetitions and frequency of these events is performed with predefined standard settings. If you want to adjust these for a particular experiment, select the **Expert** mode (only visible in **Show all**).

Parameter	Description
– Combine Software Autofocus and Definite Focus	Only available for **Tiles** or **Time Series** experiments. This strategy allows you to combine the functions of Definite Focus and Software Autofocus. This can be done in two ways, see descriptions below. Note that in both cases it is possible to modify the time point and frequency at which these events occur. The repetitions and frequency of these events is performed with predefined standard settings. If you want to adjust these for a particular experiment, select the **Expert** mode (only visible in **Show all**).
– Use Z Values/ Focus Surface defined in Tiles Setup	Only available if you have licensed the **Tiles & Positons** module. This strategy is selected automatically when the **Tiles** dimension is activated and no previous strategy was selected i.e. **None**. In the **Tiles & Positons** module (optional module), a focus surface can be defined in two ways: **Local** (for tile regions and/ or positions) or **Global** (based upon a carrier e.g. petri dish, slide, plate).
Software Autofocus as Reference for Definite Focus	Only available if **Combine Software Autofocus and Definite Focus** is selected. Software Autofocus moves the focus drive to the focus position that has been calculated. Taking this as the starting point, a new reference distance is defined for the next distance stabilization performed by Definite Focus. This can reduce the likely-hood of a stabilization failure when the sample is long and elongated and the carrier possibly tilted.
Definite Focus as Start for Software Autofocus	Only available if **Combine Software Autofocus and Definite Focus** is selected. The last valid Reference Z-Position defined by Definite Focus is the starting position for the Software Autofocus search. This allows you to optimize the search range and step size of Software Autofocus.
Initial Definition for Z Values/ Focus Surface	By default this is given by the **Tile Setup** (user defined values from the **Tiles** tool) from the Support Points / Positions / Tile regions list. It is optionally possible to use the **Software Autofocus** function or the Definite Focus. The **Recall Focus** function initially defines these value prior to the start of the image acquisition. The resulting z-values can overwrite the existing listed values in the support points/ positions list.
Z Values/ Focus Surface	
– Local (per Region/Position)	**For Tile Regions:** A local focus surface can be defined for a Tile region in two ways. ▪ For large tile regions or samples in which the plane of interest ("focus") is described by a slope or parabolic you can define one or more "support points" for each tile region. These can be arranged in a regular or irregular manner. Each support point is assigned a discrete z-value ("focus"). From these the software is able to interpolate a local focus surface with a chosen degree of complexity in an attempt to describe the contour of the plane of interest for the Tile region. ▪ When no support points are used the z value assigned to the Tile region defines the "focus" for all the tiles it contains. For small tile regions on a suitable sample this might be sufficient.

Parameter	Description
	For Positions: For positions the local focus surface is defined by the discrete Z-value assigned to it. A position cannot have support points.
– Global (Carrier based)	A global focus surface is defined based on a selected carrier template. To create a global focus surface you need to add support points by creating or editing a carrier template from the appropriate section of the Tiles tool. Thus, a group of support points are used to help describe the tilt or curvature of the carrier (again by a process of interpolation). Tile regions or position "placed" upon this global focus surface are mapped onto it accordingly.
Adapt Z Values/ Focus Surface	**Activated:** Adapts the focus surface / z-values by the following options (if available):
– Definite Focus / Software Autofocus	Here you select if you want to adapt the settings by using Definite Focus or Software Autofocus (SWAF). Depending on this selection and the available dimensions of your experiment the following additional functions can be selected from the second dropdown list:
– SWAF / As additional action	The **Reference Z-Position** calculated from the Local Focus Surface is used as the starting point for an additional software autofocus search which updates the Reference Z-Position. This allows you to reduce the search range and/or step size of the Software Autofocus (faster).
– SWAF / Update with single offset	This option is only available for **Time Series** experiments. This option allows a focus surface (local or global) to be updated by the activity of a software autofocus run. The focus surface is regularly adjusted by means of a software autofocus search which is performed exclusively at a single defined waiting position. A resulting correction of the **Reference Z-Position** is adopted for all focus areas. This strategy is only relevant if **Tiles** and **Time Series** experiments are combined. Again the repetition and frequency of the software autofocus stabilization can be modulated to meet the experiment needs.
– Definite Focus / As additional action	The Reference Z-Position calculated from the Local Focus Surface is used as the starting point for a Definite Focus stabilization, which updates the Reference Z-Position (adjusts this to a single stabilization offset, defined at the start of the experiment such that the distance between the coverslip and objective will be the same). This method only makes sense in the context of a very thin sample, where the sample lies at a constant close distance to the interface. For Definite Focus. This also reduces the likely-hood of a stabilization failure.
– Definite Focus / Update with single offset	This option is available for **Time Series** experiments only. This option allows a focus surface (local or global) to be updated by the activity of a Definite Focus stabilization. The focus surface is regularly adjusted by means of a definite focus stabilization, which is performed exclusively at a single defined waiting position. A resulting correction of the Reference Z-Position is adopted for all focus areas. This strategy is only relevant if **Tiles** and **Time Series** experiments are combined. Again the repetition and frequency of the definite focus stabilization can be modulated to meet the experiment needs.

Parameter	Description
– Definite Focus / Update with multiple offsets	This option is only available for Definite Focus.2. This approach allows a focus surface (local or global) to be updated by the activity of a Definite focus stabilization with multiple unique offsets. These unique stabilization offsets are determine immediately before the start of the experiment so that the z-values of all the support points/ positions/ tile regions are adapted individually respective to their initial z-value. This strategy can be applied to a tiles experiment with or without a time series dimension. The repetition and frequency can only be adjusted for the time series loop.
Stabilization Event Repetitions and Frequency	Only available for **Definite Focus** in combination with **Tiles** or **Time Series** experiments.
- Standard	This mode uses default settings for stabilization which we recommend to use if you are not familiar with the Definite Focus device.
- Expert	This mode allows advanced settings for using Definite Focus stabilization. Under **Synchronized with Image Acquisition** you can select how Definite Focus is used: ▪ **Time Series** If activated, this setting repeats Definite Focus at certain predefined points within a image acquisition loop (e.g. every second time point). ▪ **Tile Regions / Positions** If activated, this setting repeats Definite Focus at certain **Region / Positions** (e.g. every second position). Optionally, you can also select to run the stabilization for each tile region at either the center (**Center of Regions**) or the first acquired tile of the region (**First Tile of Regions**). ▪ **Tiles** If activated, this setting repeats Definite Focus at a certain **Tile** (e.g. every second Tile) Under **During Time series interval** you can enable Periodic Stabilization. **Periodic Stabilization** is available for experiments that include **Time Series** only. If activated, a stabilization with a defined **Period** (e.g. every 10 s) will be performed. This mode is useful if long intervals are needed between image acquisition loops. This mode can be combined with the stabilization events before discrete imaging loops.
Reference Channel and Offsets	Displays a table with the currently defined channels. The column **Offset** sets an offset in µm. The button **Set as Reference Channel** sets the currently selected channel as reference.

For more information on focus strategy, see *Introduction [▸ 86]*.

13.3.2.5.1 Focus Strategy Wizard

This wizard guides you through the setup of a suitable focus strategy for your experiment. Currently this wizard is only available to help you optimize the focus strategy for a tiles or positions experiment. Therefore, the **Tiles & Positions** license is necessary for this wizard.

General Wizard Controls

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard. No changes are applied to your focus strategy settings.
Finish	Saves the setup and the changes based on your progress and closes the wizard.

13.3.2.5.1.1 Preparation Step

This step serves as a preparation for the setup.

Parameter	Description
Best Practice Checklist	Displays a list of best practices to consider before continuing with the setup.
Dimensions	Displays the currently configured dimensions of your experiment. To change the dimensions in your experiment you have to leave the wizard and change them on the **Acquisition** tab.
Reference Channels & Offsets	
– Name	Displays the name of the experiment channels.
– Offset (µm)	Sets and displays the offset for each channel. The reference channel is used by the software autofocus. If your desired focus in the specimen should be offset to the result of the software auto focus, enter the relative values (in µm) here. It can be defined for each channel (fluorophore) in the image independently. Offsets can be used without the software autofocus to create a relative difference in focus for any channel to the reference channel. Offset values can be determined outside the wizard. This is a manual process specific for your given sample and application. Values in the **Channels** tool or **Focus Strategy** tool will appear here and can be modified.
– Set as Reference Channel	Sets the currently selected channel as reference channel.
Objective	Only available if a Definite Focus is configured. Shows the selected objective and if it is compatible with Definite Focus (if DF 2 is configured).

13.3.2.5.1.2 Determine Initial Focus Values Step

This step helps you to determine initial focus values for you experiment. You can use the *Software Autofocus [▸ 717]*, the *Definite Focus [▸ 717]*, or you can do it manually in the **Tiles** tool.

When you create/ add a position or tile region, it is always assigned a z-value that corresponds to the current focus position. You can manually adjust these values directly in the respective section of the *Tiles tool [▸ 531]*. For more information, see also *Adjusting Z-Values [▸ 520]*. Alternatively, you can click on the **Verify** button to open the *Verify Tile Regions or Verify Positions Dialog [▸ 542]* which helps you adjust the values.

13.3.2.5.1.2.1 Determine Initial Focus Values (Software Autofocus)

If you select to apply the Software Autofocus (SWAF), ZEN uses the SWAF to determine the z-values for positions, tile regions or their support points. Make sure that your sample is suitable for use of the SWAF which uses the settings of the reference channel.

Every experiment in ZEN blue has SWAF settings. They can be seen and modified in the *Software Autofocus Tool [▸ 507]*. The default settings are usually a good starting point, but should be optimized according to the sample. You can find a detailed description of the parameters in the help chapter for the tool. The adjustments will help reduce the time it takes to run the search but still reliably determine the focus.

See also

Determine Initial Focus Values Step [▸ 717]

13.3.2.5.1.2.2 Determine Initial Focus Values (Definite Focus)

If you select to apply Definite Focus.2 (DF.2), ZEN uses the DF.2 device on your Axio Observer to determine the z-values for positions, tile regions or their support points. Make sure that the selected objective and your sample are suitable for use of the Definite Focus.2.

In the first step of this wizard, ZEN indicates if the selected objective can be used. You should test your sample with DF.2 beforehand, but typically the following guidelines can be given:

- Sample with coverslip glass or glass bottom (**suitable**)
- Objective type = air/dry lens and sample fixed (**suitable**)
- Objective type = oil immersion and sample fixed (**not suitable**)
- Objective type = oil immersion and sample aqueous media i.e. cells in culture (**suitable**)
- Objective type = water immersion and sample fixed or aqueous (**suitable**)

Definite Focus uses a difference of the refractive index to detect the position of the objective relative to the sample/ cover slip surface. This position can be stored and recalled quickly with high resolution (accuracy) and reproducibility (precision). You can also define an offset to this position by focusing into the sample as required and clicking on **Store focus** in the *Definite Focus Tool [▸ 802]* (right tool area).

Definite Focus.2 can be used to setup the z-values of your positions, tile regions or their support points. It is considerably faster than using a Software Autofocus. Keep in mind that this approach will adjust the all z-values according to the local topology of the sample surface relative to the objective. Thus, your sample should be in focus if it follows this topology.

See also

Determine Initial Focus Values Step [▸ 717]

13.3.2.5.1.3 Sample Size and Distribution Step

This step determines which focus surface is needed for the experiment based on the sample size and distribution. For general information, see *Focus Surfaces in ZEN [▸ 718]*. The first two options require a local focus surface, which can be employed with or without a sample carrier. If your sample corresponds to the third option, a global focus surface is necessary, which can only be used and modified with a sample carrier template.

Parameter	Description
Sample Carrier	Selects a sample carrier and displays the name of the currently selected one.
– Select	Opens the *Select Template Dialog [▸ 557]* to select a sample carrier.
– Edit Support Points	Opens the sample carrier selection/editor dialog. Here you can edit and add global support points to the selected sample carrier.
– Delete	Deletes the selected sample carrier from the sample carrier field. The template will still be available in the **Select Template** dialog.
Move Focus to Load Position Between Container	Only visible if a sample carrier is selected. **Activated:** The focus drive moves to the load position while moving to another container of the sample carrier (e.g. a well or a slide). This can prevent possible damage/collisions with obstructions that occur between different areas of the sample carriers underside.

13.3.2.5.1.3.1 Focus Surfaces in ZEN

Depending on the nature of your sample, its size, and its location on the sample holder, ZEN provides two options that employ a so called focus surface to help keep your sample in focus. A focus surface is basically a topographical map of a certain part of the sample. Like the contour lines on a map, this tells the microscope for any XY coordinate on it how the objective should be set in Z in order to make sure the image of the sample is sharp.

In principle, ZEN uses the same method regardless of the size of the sample. A focus surface can simply be a single z-value assigned to your position or small tile region. For larger more complex objects it is more sophisticated. For example, large tile regions created to image an entire tissue section might have several hundred images that make it up. It is not plausible to have a single z-value that defines the focus, neither is it efficient to define the z-value at each of these several hundred images. In this case the focus surface is defined by a limited number of z-values at discrete locations. The z-values of these “support points” are used to create a topographical map – the focus surface - by interpolation. It covers the entire region you want to image and defines the focus of a single image acquired on it. In this way huge areas can be imaged where every frame is focused correctly.

ZEN defines two types of focus surface:

- **Local**: Use this mode to image groups of positions, small tile regions or mixtures of both at high and lower magnifications. It is also used to image large tiles or a number of large tiles i.e. tissues sections or embryos. This is the standard setting for most imaging applications.

- **Global**: Use this mode to image samples that are widely spread across your sample carrier. The global focus surface is assigned to and defined on your sample carrier template. In this manner it is typically used at lower magnifications when a very precise local z-value is not needed to create a sharp image. In some cases this allows it to be used for more than one sample carrier after another if they are mounted in a regular and consistent manner.

See also

Sample Size and Distribution Step [718]

13.3.2.5.1.4 Adapting Focus Values Step

This step is only available if at least a DF.2 or SWAF is configured. You are asked if you want/need to adapt the focus during your experiment. If you have both Definite Focus and Software Autofocus configured, you are also asked if the focus is static relative to the sample carrier. Depending on your configuration and selection, the adaptation of focus values via Definite Focus or Software Autofocus is displayed in the next step of the wizard.

13.3.2.5.1.4.1 Adapting Focus Values in ZEN

Adaption of the focus values in your experiment might not always be necessary. Typically, if you have followed the suggestions laid out at the beginning of the Focus Strategy wizard for best practice and your experiment does not involve creating time lapse images of the specimen or long extended periods of imaging (i.e. the sample is fixed or no longer living), you might not need to adapt the focus at all during the imaging process. The z-values or focus surface values you define will probably be stable enough that the images of the specimen will remain sharp.

However, in many cases, especially when living samples are involved, it is necessary to adapt your z-values. Depending on the configuration of the system two approaches are available to adapt your z-values:

- **Software Autofocus**: This is a technique that adapts the focus based on taking a series of images at planes above and below the current z-value. The system evaluates these images based on contrast or intensity and then determines where the highest value lies. This plane becomes the new z-value and in most cases it is the plane we are interested in or can be used as reference for it. Z-stacks are centered relative to this value. This allows an adaptation of z-values when the sample moves (e.g. growth, migration), but also if the sample and sample carrier drift together. By its very nature this approach might need optimization to get better, more reliable, and/ or faster results.
- **Definite Focus.2**: This is a function to quickly determine the distance between your objectives front lens and the sample. Using DF.2, only the drift of the sample and sample carrier together can be adapted. This can be done to restore or maintain this distance anywhere and at any time. If necessary you can adjust this distance depending on the location in the sample and how far into it the focus is required. Adaptation of the focus with Definite Focus.2 is very quick and highly repeatable. As it is based on the physical principles of light refraction, it requires certain characteristics of the sample and objective. ZEN informs you if the objective is not suitable and adjusts the selection in the Focus Strategy wizard accordingly.
  See also the chapter *Determine Initial Focus Values (Definite Focus) [717]* which gives some basic guidance on the types of sample that can be used with the different objective types.

See also

Adapting Focus Values Step [719]

13.3.2.5.1.5 Adapting Focus Values with Software Autofocus Step

This step guides you through the adaptation of focus values with the Software Autofocus. The options displayed here depend on whether the experiment is a time series or not, and also on the type of sample which you have selected in the *Sample Size and Distribution Step [▸ 718]*.

13.3.2.5.1.6 Adapting Focus Values with Definite Focus Step

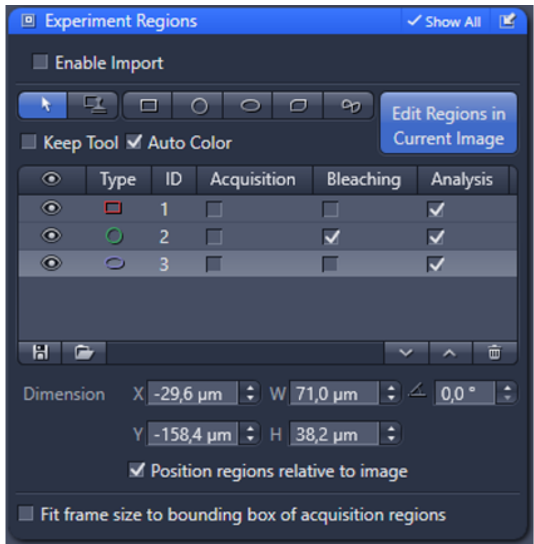
This step guides you through the adaptation of focus values with the Definite Focus. The options displayed here depend on whether the experiment is a time series or not, and also on the type of sample which you have selected in the *Sample Size and Distribution Step [▸ 718]*.

13.3.2.5.1.7 Manually and Interactively Setting Focus Values Step

This wizard step provides you with detailed information on how to set focus values manually. The displayed information depends on the sample characteristics you have selected in the *Sample Size and Distribution Step [▸ 718]*.

13.3.2.6 Experiment Regions Tool

This tool allows to define Regions of Interest (ROIs) which are used for image acquisition, sample manipulation (bleaching) and image analysis.
If you are using FCS for LSM 980, see the chapter *Overview Acquisition Tools [▸ 918]* for FCS specific options in the **Experiment Regions** tool.



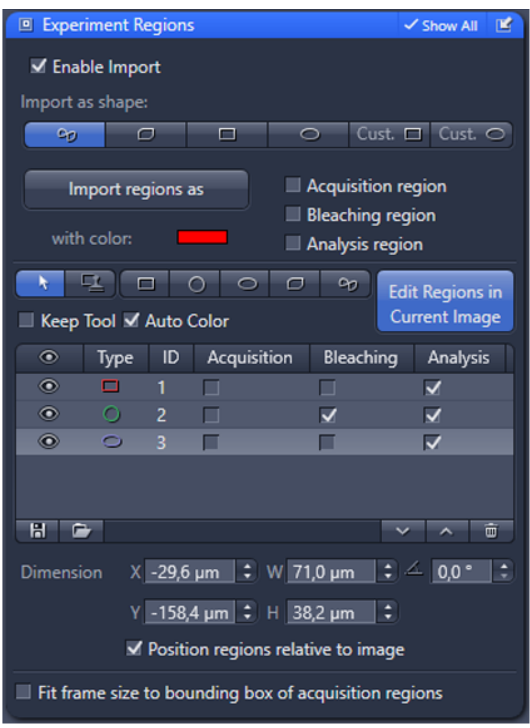


Fig. 66: Experiment Regions Tool (Show All) - Enable Import deactivated and activated

Parameter	Description
Enable Import	Activate this option to show additional controls for the import of Regions which were created by an Image Analysis workflow upfront. This option is only available in **Show all** mode.
Import as shape: Toolbar	Select here, which type of Shape the imported. The shape of the created Experiment Region can either be a polygon, a simplified polygon or a rectangle or ellipse with the approximate size of the bounding box from the analysis region. Alternatively you can define a custom rectangle or ellipse with specific size and an offset relative to the center of the bounding box of the analysis result.
Import regions as	When you press this button, the currently selected regions from an Image Analysis result will be imported into the list of Experiment Regions. The properties, e.g. if the regions will be used for bleaching and analysis, are defined by the neigboring checkboxes.
Color	Select here the color which will be used to indicate the imported regions in the image.
Toolbar	Use the tools from the toolbar to draw ROIs into the image.
Edit Regions in Current Image	To add a new Experiment Region to your experiment, you need to click the **Edit Regions in Current Image** button first. The button will turn to blue to indicate that Experiment Regions are displayed in the image and can be added or manipulated. When the button is not enabled, all standard graphical elements are shown in the image, but not Experiment Regions. Standard graphical elements are used for image analysis and annotation, but will not have an impact on the next image acquisition. Experiment regions which are used e.g. for bleaching, are automatically converted into standard graphical elements while the experiment is performed. It is not possible to edit Experiment Regions when the image is displayed in certain viewers. Switch back to the 2D view, in case the button is disabled.
Keep Tool	**Activated**: Keeps the selected graphic tool for multiple actions.
Auto Color	**Activated:** Assigns different colors for each drawn ROI. Only available in Show all mode
ROIs List	This list shows all drawn in ROIs . Their **Type** (form and color), **ID** (number) are listed as identifiers. For each ROI certain properties can be chosen by activating the according checkbox:
- Acquisition	**Activated:** Acquires images only within the ROI(s). Use this parameter in combination with **Fit frame size to bounding box of acquisition regions**.
- Bleaching	**Activated:** Performs a bleaching experiment will be within the selected ROI(s). The bleaching parameters are specified in the **Timed Bleaching Tool**, sse chapter *Timed Bleaching Tool [▸ 762]*.

Parameter	Description
- Analysis	**Activated:** Uses data for Mean of ROI analysis in the **MeanROI** view from the corresponding ROI(s) .
Save	Saves the selected ROI(s) to the file system.
Load	Loads ROI(s) from the file system.
Delete	Deletes the selected ROI(s) from the ROIs list.
The following parameters are only visible in the **Show All** mode.	
Dimensions	
- X / Y	Shows the X / Y position of a selected ROI. Enter new values in the input fields.
- W / H	Shows the Width (W) and Height (H) of the selected ROI. Enter new values in the input fields.
- Angle	Shows the rotation angle of the selected ROI. Enter a new value in the input field.
- Position regions relative to image	The experiment region is placed in relation to the current field of view. Use this option if the regions should maintain their position within the image in case the stage is moved. When this option is deactivated, the regions are defined by their stage coordinates. In this case, regions can be placed on certain structures of your sample and the regions will stay there when the stage is moved. Note, that the system will not attempt to automatically move to these positions e.g. during bleaching experiments, if they are not accessible in the current scan area.
Fit frame size to bounding box of acquisition regions	**Activated:** Fits all ROIs that are marked as Acquisition ROIs in the table to the total frame that the scanner will cover. This can decrease imaging time, since the scanner has not to move over the complete frame of the original image, in which the ROIs were drawn. While the frame size is reduced, the scan speed will maintain its value as configured in the Acquisition Mode tool. In order to achieve maximum acquisition speed, you should use the **Acquisition Mode Tool** to manually reduce the frame size and adjust the speed correspondingly.

13.3.2.7 Experiment Designer Tool

Info

This tool is only visible if you have licensed and activated the module in the **Modules Manager**. Additionally you must activate the corresponding checkbox on the **Acquisition** tab in the **Experiment Manager**. This tool is part of the basic license for LSM.

In the **Experiment Designer** you can create experiments for multidimensional acquisition. Experiments can consist of any number of components. A component is referred to as an experiment block. Each experiment block has a distinct number, which is shown above the block. To create a new experiment block click on the **Create Acquisition** button and select the desired type of block (Acquisition, Delay, Wait, Execute blocks) then click on the button again. The block will appear in the **Timeline** of the experiment which you see below the blocks.

Please note the following when working with experiment blocks:

- Each acquisition block can be seen as its own independent single experiment with its own individual settings.
- Each experiment block can have its own dimensions (e.g. channel settings like exposure time, active camera, camera parameters; Z; T).
- Focus strategies are block specific as well.
- You can change the order of experiment blocks via drag & drop in the experiment timeline.

Special actions that influence the course of an experiment are performed by means of a special block. In the **Show All** mode you can define Loops and Repetitions and specify the number of image files.

Parameter	Description
Import	Opens the *Import experiment blocks from other experiments Dialog [▸ 724]*. Here you can choose blocks to import from existing experiments.
Export	Opens the **Export Experiment Blocks** dialog. You can choose here, which experiment blocks you want to export to to the file system.
Create Acquisition Block	Adds a new, empty acquisition block to the experiment timeline.
Create Delay Block	Adds a Delay block to the experiment timeline. A delay block pauses the experiment for a predefined period. After that period the experiment continues automatically. You can set the length of the pause in the **Properties** section with the **Delay** slider or input field by clicking on a Delay block. The delay will be shown within the block. If the **Synchronize with preceding blocks** checkbox is activated, the duration of the delay block is reduced by the measured execution time of the preceding blocks.

Parameter	Description
Create Wait Block	Adds a Wait block to the experiment timeline. A wait block holds the experiment in idle status as long as clicking on the **Continue Experiment** button in the message box. This can be used for adding a solution or changing the buffer of the specimen. The message box will be shown when a wait block is reached. You can enter a message text into the input field in the **Properties** section by clicking on a **Wait!** block.
Create Execute Block	Adds an Execute block to the experiment timeline. A execute block executes a selected hardware setting. You can change the blocks properties by clicking on the corresponding block. If the **Sequential** checkbox is activated the experiment will continue while the hardware setting is executed. If it is deactivated the experiment will wait until the hardware setting was executed. By clicking on the *Options [▸ 725]* button the options for configuring and selecting the hardware settings will appear. If a hardware setting was selected, the **Go!** button is active. If clicking on this button the selected hardware setting will be applied immediately.
Duplicate	Duplicates the selected block and inserts the newly created block after the last block.
Delete	Deletes the selected block from the timeline.

13.3.2.7.1 Import experiment blocks from other experiments Dialog

Here you can import experiment blocks from existing experiments.

Parameter	Description
Choose Experiment	Here you select the experiment you want to import experiment blocks from. The experiment must have been saved on your computer before.
Select desired blocks	Here you can select the experiment blocks you want to import. Simply click on an experiment block to select it. The selected block is marked in blue. If you want to import all blocks of an experiment don't select a block but continue by clicking on the **Import** button directly. This will import all experiment blocks at once.
Import	Imports the experiment or the selected experiment blocks to the experiment timeline.
Cancel	Cancels the import.

13.3.2.7.2 Create Execute Block Options

Parameter	Description
Edit Setting/Light Path	Opens the light path dialog in which you can change the relevant hardware setting.
Set to None	Removes the existing hardware setting.
Get Current Hardware	Adopts the current device status.
Experiment Settings Pool	The shortcut menu shows a list of the existing hardware settings.
Harddrive Folder	The shortcut menu shows a list of the saved hardware settings.
From File...	Opens the Import Hardware Settings dialog window. Select a ZIS hardware settings file (*.czhws).
Export to User Folder	Exports the current hardware setting.

13.3.2.7.3 Loops and Repetitions section

Only visible if the **Show All** mode is activated.

Here you can specify which experiment blocks should be repeated during the experiment. You can define as many repetitions as you like for each experiment. An experiment block may only appear once within the repetitions defined.

Info

If you define several repetitions, the following conditions must be met:

- Repetitions must form a complete unit.
- One repetition may not be placed within another.

If these conditions are not met, the repetition cannot be performed. In this case a yellow warning symbol appears under the **Active** field.

Parameter	Description
Loops	Enter the number of loops that you want to be performed.
Start	Enter the number of the starting block.
End	Enter the number of the end block.
Active	If the checkbox is activated, this repetition is performed in the experiment.
Add	Adds a new repetition to the experiment.
Delete	Deletes the selected repetition.

13.3.2.8 Z-Stack Tool

Info

The basic **Tiles** tool is only visible if you have a motorized stage configured with your microscope. The **Tiles Advanced Setup** and many other functions are only available if you own the **Tiles a& Positions** module and when it is activated in the **Modules Manager**. Additionally you must activate the corresponding checkbox on the **Acquisition** tab in the **Experiment Manager**. This tool is part of the basic license for LSM.

In the **Z-Stack** tool you can configure acquisitions that comprise several Z-planes of your sample. You can set all the parameters manually using two different modes (see *Manual Configuration* [▸ 728]) or have configuration performed automatically (see *Z-Stack Automatic Configuration* [▸ 727]).

Z-Stack Graphical Display

The graphical display in the left area of the tool represents the configured Z-stack. In the case of inverse microscopes the objective appears in stylized form at the bottom of the Z-stack. In the case of upright systems it appears at the top.

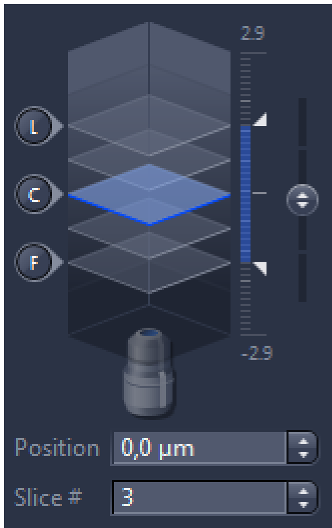


Fig. 67: Graphical Display of the Z-Stack

The blue plane indicates the current section plane. The round **L**, **C** and **F** buttons refer to the corresponding planes (**L** = Last, **C** = Center, **F** = First). To change the current Z-position, click on the relevant buttons. The blue plane then jumps to the desired position.

The values at the top and bottom of the measurement scale on the right-hand side of the graphic indicate the distance to the center of the Z-stack.

The **Position** display field below the graphic indicates the Z-position at which the section plane is located. Here you can navigate precisely to the relevant Z-positions.

The **Slice #** display field below the graphic indicates the number of the current slice.

13.3.2.8.1 Z-Stack Automatic Configuration

Info

Before you perform automatic configuration, the current focus position must be at the center of the sample. The camera's current field of view must always be at a position on the sample that shows a signal in the selected channel.

If you click on the **Z-Stack Auto Configuration** button of the tool the automatic configuration is performed.

Note that Z-stack automatic configuration only works with microscopes and systems that do not use an optical sectioning technique. If you use an **LSM**, **ApoTome**, **VivaTome**, **Spinning Disc (CSU)** or another technique for generating optical sections, the Z-stack must be configured manually.

The following parameters are set automatically:

- Z-position of the central plane
- Distance between the individual planes
- Number of section planes

Parameter	Description
Start Auto Configuration	Automatically configures the Z-stack using the current sample.

13.3.2.8.2 Manual Configuration

Info

Z-Stack images are always acquired from bottom to top automatically, irrespective of whether you have defined the top or bottom z-plane of your stack as the first z-plane. This acquisition sequence increases the accuracy of the z-positioning.

For manually configuring z-stacks you have two modes available. Please note that these modes are only available if **Show All** is activated.

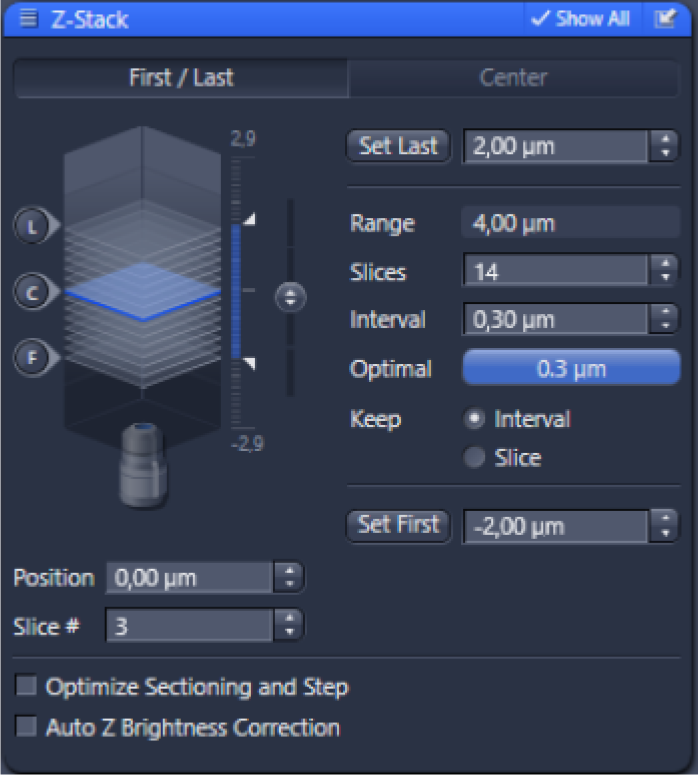


Fig. 68: Z-Stack Tool (Show All activated)

Parameter	Description
First / Last Mode	If activated, you are able to configure the z-stack via setting the first and the last positions of the z-stack, see *Configuring a Z-Stack manually (First/Last Mode) [▸ 48]*.
Center Mode	If activated, you are able to configure the z-stack via setting the center plane of the z-stack, see *Configuring a Z-Stack manually (Center Mode) [▸ 48]*.

Depending on which mode you have activated, you will see the following parameters for configuring the z-stack:

Parameter	Description
Set Last/Set First	Only visible for **First/Last** mode. By clicking on the **Set Last** and on the **Set First** button you determine the current position as last or first position of the z-stack.

Parameter	Description
Range	Displays the range of the configured z-stack from the last to the first section plane.
Slices	Here you can enter the number of z-slices that the z-stack will have.
Interval	Here you can enter the desired distance between the z-slices.
Optimal	The number on this button shows the distance calculated for the channels set and the current microscope according to the Nyquist criteria. If you click on the button, this value is automatically adopted into the **Interval** input field. When you click this button once, it will change its color to blue. This indicates that the system will always use the optimal interval as you continue to change acquisition parameters. Click the button again or manually edit the interval to deactivate this permanently active state. **Note:** If you change the objective while the button is activated, the value might not be updated correctly!
Keep	▪ **Interval**: Keeps the set interval between the section planes constant if you change configuration parameters in the Z-Stack tool. ▪ **Slice**: Keeps the set number of z-slices constant.
Center	Only visible for **Center** mode. If clicking on this button the current position is set for the central z-plane. You can also enter the value in the input field to the right of the button.
Offset	Here you can enter a value for an offset if desired.
Optimize Sectioning and Step	This function is for LSM only. The tool allows to set the pinhole of each track to a diameter such that the section thickness of each track is identical. This can only be achieved if the tracks are switched in Frame mode during acquisition allowing hardware to be changed between tracks. Each channel is represented by a graphical display of two z-slices. The

Parameter	Description
	channel name, the z-slice thickness and the current overlap of two z-slices in percent are given above the graphic.
- Match Pinhole	Changes the pinhole of the tracks to match the smallest available section thickness. Hence to go for a specific section thickness identical for all tracks, set the pinhole of one track to achieve the desired section thickness and open up the pinhole of all other tracks to maximum. Then click on **Match Pinhole** to get the right settings for all tracks. In case the step interval is subsequently changed to lower or higher settings, clicking **Match Pinhole** changes the pinhole size for all tracks to achieve again 50% overlap between the single steps in each track.
- Optimal	Sets the interval for all tracks to the optimal value. The optimal value is based on the current smallest available section thickness of the current tracks which is defined by the pinhole settings.
- Undo	Resets all parameters to the default values.
- X:Y:Z = 1	Matches the settings in Z to the settings in X and Y. This produces a cubical voxel. This can be useful for later import into third party software for rendering.

The settings of **Auto Z Brightness Correction** are part of the image acquisition and are reused with other settings of an image. They are also part of an experiment setting. However, the function is not activated for reuse or when loading an experiment as the settings apply to the absolute z-position in µm used when the (previous) image was acquired. If the new stack is acquired in a different position, using the previously defined settings (extrapolate) can lead to a an extreme overexposure of the sample. Enable test will always be deactivated when an image stack is reused or an experiment is loaded.

Worflow:
In order to reuse the Auto Z Brightness parameters for subsequent z-stacks make sure to manually set the center (or first or last) position of the first z-stack to zero (Focus TW, Z-position: Set Zero) before defining the Auto Z parameters. Set the center (or first or last) position of all following Z-Stacks also to zero to be able to reuse the Auto Z Brightness parameters accordingly. When saving and loading the parameters, the same logic applies. When switching between linear and spline interpolation during continous scan, the current acquisition parameters are not updated until the z-position is changed.

Parameter	Description
Auto Z Brightness Correction	This function is for LSM only. If activated, certain acquisition parameters (Laser power, Master gain, Digital offset and Digital gain) are automatically adjusted according to the predefined settings while the Z-Stack is being acquired. Note that Z Piezo is not used for Z stack acquisition when the master gain should be adjusted with z position. The gain adaptions take too long to be set between z slices. When indicating a change in master gain the slower z focus function of the microscope is used instead. The settings are typically adjusted while focusing through the sample when the system is in continuous scan mode. Activate the option **Enable Test** to allow an update of the settings during focusing either with the focus control of the microscope or using ZEN. ZEN does not show the update of the values during the acquisition of the experiment. Note that resetting the focus position in the **Focus** tool does not affect or shift the absolute position values in the list.
- Add	Adds the current Z-position to the list and stores the currently configured settings. If the position is already in the list, the values are updated. The positions do not need to be added in a certain order.
- Move to	Changes the focus position to the selection in the list. This can also be done by a double click on the list item. If **Enable Test** is activated, the parameters are immediately applied during a continuous scan.
- Remove	Removes the currently selected position from the list.
- Remove All	Removes all positions from the list.
- Load../Save..	Loads or saves the stored position parameters to/from a *.ABC file.
- Spline Interpolation	If activated, a spline interpolation instead of a linear interpolation is used. Note that spline interpolation with only few positions in between the neighboring images can show an overshoot of the applied acquisition settings and hence lead to very bright or dim images.
- Extrapolate	If activated, the interpolation between the Positions in the list can be extrapolated to the actual first and last slice of a z-stack in case if those are not part of the defined range of Positions.
- Enable Test	If activated, the parameters are updated while changing the current z-position either with the SW focus or with the handwheel of the stand. This allows to quickly check the parameters during a continuos scan. The changes of the parameters are not indicated during the actual experiment.

13.3.2.9 Time Series Tool

Info

This tool is only visible if you have licensed and activated the module in the **Modules Manager**. Additionally you must activate the corresponding checkbox on the **Acquisition** tab in the **Experiment Manager**. This tool is part of the basic license for LSM.

Info

If you define times as timer series acquisition conditions, these apply once for the entire experiment. This also applies to experiments that use the **Experiment Designer**

Use Time Series to acquire an image series consisting of a number of time points. Define, for example, the acquisition interval, the length of the experiment and other specifications to control the experiment.

Parameter	Description
Duration	Defines the minimum duration of your experiment with the slider or the up and down arrows. The drop-down list defines the number of time points in cycles or the duration: ▪ **Cycles** ▪ **ms**: milliseconds ▪ **s**: seconds ▪ **m**: minutes ▪ **h**: hours ▪ **d**: days
As Long as Possible	**Activated**: The acquisition of the time series continues as long as possible depending on the free disk space of the hard disk. That means the following: ▪ For hard disks with **more than 20 GB free disk space** the acquisition always runs until **2 GB disk space** is left. **Example:** An experiment using a hard disk with 250 GB of free disk space will run until 2 GB are left. The calculated/required disk space for the experiment is 248 GB. ▪ For hard disks with **less than 20 GB free disk space**, at least **10 % disk space** will be always left free. **Example:** An experiment using a hard disk with 15 GB of free disk space will run until 1,5 GB are left. The calculated/required disk space for the experiment is 13,5 GB.
Interval	Defines the interval from individual image to individual image in an image series. You can specify the interval to set the gap between individual time points in milliseconds, minutes, hours or days. For LSM it is possible to change the Interval during timer series acquisition with the Parameter Interval Time.

Parameter	Description
Use Camera Streaming if Possible	Only visible in **Camera/Widefield** Mode. **Activated**: The software tries to use the free running/streaming mode of the active camera.
Use Burst Mode if Possible	Only visible in **Camera/Widefield** Mode. **Activated**: The system buffers acquisition data in the main memory. For more information, see Burst Mode.
Measure Speed	Not available for **LSM** acquisition. Checks whether the experiment can be performed using the interval which is set. If the interval is too small, the shortest possible value is defined automatically for the interval.
Start / **Start Next Time Slice** / **Stop** / **Pause Begin** / **Pause End**	Defines the **Start**, **Stop** and **Pause** conditions of your experiment. Select the parameters for the corresponding condition from the **Mode** dropdown list.
– **Manual**	The experiment is started immediately when clicking on the **Start Experiment** button in the **Experiment Manager**.
– **At Time of Day**	The experiment is started, stopped or paused at the entered time. Enter the desired time in the spin box / input field below the dropdown list.
– **After Delay**	The experiment is only started, stopped or paused once the length of time entered has passed.
– **On Trigger**	The experiment is started, stopped or paused once a TTL signal has been received.
Interval Time	This function is only available for LSM 980. Use this function to interactively change the interval during a running time series acquisition.
– **Interval**	Adds a new interval value which is taken over from the current set interval. To add a new interval, simply click on the **Add** button.
– **Trigger In**	Assigns a **TriggerIN** signal to change the interval.
– **Trigger Out**	Assigns a **TriggerOUT** signal for each interval change
Set Marker	This function is only available for LSM 980. Use this function to mark events during the time series acquisition. To define a new marker, simply click on the **Add** button . The marker is applied within the acquired time series when clicking the **Set** button or via trigger signal.
– **Description**	Predefine the marker by typing in the marker name into the editing box.
– **Set** **Trigger In**	Click **Set** or use **TriggerIN** to set a marker during time series acquisition.

Parameter	Description
– **Trigger Out**	If necessary, assign a **TriggerOUT** signal for every set marker. Note that the **TriggerOut** signal for the marker function consists of a high spike followed by a 3 second long TTL pulse. Within the duration of the pulse a further low spike is delivered.
Interactive Switches	Adds and configures switches that can be used to execute certain actions during your experiment. To add a new switch, simply click on the **Add** [+] button. The Switch button opens a dialog to configure an interactive switch. For more information, see *Interactive Switches section [▸ 735]*.

Info

The shortest possible interval is calculated by performing a blind experiment. The camera exposure time, number of steps of a Z-stack and the number of acquisition channels are taken into consideration in the calculation. Depending on the number of Z-stacks and channels and whether long exposure times have been set, it may take some time to calculate the shortest time interval.

13.3.2.9.1 Experiment Conditions section

The following functions are only visible if the **Show All** mode is activated:

You can define the **Start**, **Stop**, **Pause Begin** and **Pause End** conditions for your experiment. **Start Next Time slice** can only be used with trigger functionality of the LSM 980. Select the parameters for the corresponding condition from the dropdown list:

Parameter	Description
Manual	The experiment is started immediately when clicking on the **Start Experiment** button in the **Experiment Manager**.
At Time of Day	The experiment is started, stopped or paused at the entered time. Enter the desired time in the spin box/input field to the right of the dropdown list.
After Delay	The experiment is only started, stopped or paused once the length of time entered has passed.
On Trigger	The experiment is started, stopped or paused once a TTL signal has been received.
Interval Time	This function is only available for LSM 980. Use this function to interactively change the interval during a running time series acquisition. Use the **+** button to add a new interval value which is taken over from the current set interval. Add another interval first before changing the current interval. Highlight the desired interval during the experiment to change the interval before the next image frame is scanned or assign a **TriggerIN** signal to change the interval. You can always assign a **TriggerOUT** signal for each interval change.

Parameter	Description
	The change of the interval is also marked in the time series acquisition. The marker is visible next to image (2D view, Gallery view, Mean-ROI) when the change occurred.
Set Marker	This function is only available with LSM 980. Use this function to mark events during the time series acquisition. Predefine the marker by typing in the marker name into the editing box. Either click **Set** or use **TriggerIN** to set a marker during time series acquisition. You can also assign a **TriggerOUT** signal for every set marker. The **Set** button becomes active when the acquisition is running. The marker is visible next to image (2D view, Gallery view, MeanROI) when the marker was set.

Info

If you define times as timer series acquisition conditions, these apply once for the entire experiment. This also applies to experiments that use the **Experiment Designer**

13.3.2.9.2 Interactive Switches section

The following functions are only visible if the **Show All** mode is activated:

Here you can add and configure switches that can be used to execute certain actions during your experiment.

Left-click on a new or existing switch to open the dialog in which you can configure the button parameters:

Parameter	Description
Name	Here you can enter a name for the button.
Description	Here you can enter a description for the button.
Color	**Activated:** Shows a colored line at the left edge of the switch.
Color Selection	Opens the **Color Selection** dialog. Here you can select a color for the line at the left edge of the switch.
Action	Here you can select one of the following actions. This action will be executed when you click on the button: ▪ **None** ▪ **Set Interval** ▪ **As Fast as Possible** ▪ **Trigger** ▪ **Hardware Setting** ▪ **Jump to previous block** ▪ **Jump to next block** ▪ **Jump to block #**
Delete Switch	Deletes the selected switch.

13.3.2.10 Experiment Information Tool

Here you can find an overview of your experiment parameters, e.g. the memory requirement of the experiment or its duration.

Parameter	Description
Required Disk Space	Indicates the calculated memory space that the experiment will take up on your hard drive. All the activated blocks of an experiment created using the **Experiment Designer** are taken into account.
Duration (Theoretical)	The system adds together all the exposure times arising during acquisition in the experiment and indicates this value. In the case of time series the intervals set are also taken into account. The actual acquisition duration will always turn out longer, however, as switching times for components (diaphragms, reflectors) and positioning times (Z-plane, stage position) also come into play.
Maximum Acquisition Rate	If the **Time Series** acquisition dimension is activated in the **Experiment Manager**, you can measure the maximum possible frame rate of the system in the **Time Series** tool. In that case the frame rate is shown here. Otherwise "not available" is displayed. After any change is made to the experiment the frame rate must be determined again in the **Time Series** tool.
Elapsed Time (Last Experiment)	If you have already run the current experiment before on the system, the duration actually required for it is displayed here. This information disappears again if you change the experiment.
Next Time Point in	Shows duration to next time point.
Tile Size	Shows the X/Y dimensions of your experiment. In the case of a single position this value is identical to the size of the camera field.

13.3.2.11 Experiment Feedback Tool

Info

This tool is only visible if you have licensed and activated the module **Advanced Processing** in the **Modules Manager** and additionally activated the **Experiment Feedback** checkbox in the **Experiment Manager** on the **Acquisition** tab.

Parameter	Description
Edit Feedback Script...	Opens the *Script Editor [▸ 737]* dialog. There you can create scripts for an Experiment Feedback.

Parameter	Description
Select script run-time conditions	
- Free Run	Upon the experiment start the acquisition and the feedback script are started but run from here in an completely unsynchronized manner. The online image analysis or the script run itself will not slow down the actual image acquisition.
- Synchronized	This mode will lead to strictly determined order of events depending on the chosen level of synchronization. The online image analysis and/or the feedback script will be started after current acquisition is finished. In contrast to the **Free Run** mode, a synchronized run can slow the whole acquisition down. The big advantage of the mode is, that the synchronized run ensures a predictable workflow.
Define Script Slot	Here you define the experiment feedback sequence by arranging the slots (represented by blue buttons). The blue slots run one after another the non blue slots are run separately. ▪ The **Acquisition** slot represents the actual image acquisition. ▪ The **Analysis** slot represents the online image analysis. ▪ The **Script Run*** slot represents the execution of the experiment feedback loop script. Note that the loop script will be only executed when triggered by a used observable inside the loop script. ▪ The **HD Writing** slot represents the slot for writing the image data to your hard drive.
Allow additional loop script runs	Triggers the main loop if observables that are not part of the multidimensional experiment (e.g. frame index, time index, block index, etc.) change. Those observables could be time or temperature of the incubation. If the checkbox is not activated only experiment observables trigger the main loop script. This only applies when the acquisition is idle. This only applies when acquisition is idle.

13.3.2.11.1 Script Editor for Experiment Feedback Dialog

See also

Editing the Feedback Script [▸ 241]

13.3.2.11.1.1 Input Windows

The three windows on the left allow you to input scripts based on the programming language Python:

Window	Description
Pre Loop - Single Execution on Experiment Start	Import modules and define functions or variables. This part is executed only once at the beginning of the feedback experiment.

Window	Description
Loop Script - Repetitive Execution During Experiment Runtime	Executed every time an observable used within the loop script changes. Allows to modify the experiment on-the-fly and react on results from the online image analysis, e.g. stop the acquisition when a defined number of cells have been counted, or to take action upon external signals.
Post Loop Script - Single Execution on Experiment Stop	Define actions that are executed only once when the acquisition is finished, e.g. write the data in a logfile or play a sound when the experiment is finished.
Clear output	Clears the output messages field.
Accept	Adopts the script within the experiment without closing the window.
OK	Adopts the script and closes the window.
Cancel	Leaves the dialog without adopting the script.

For more information, see *Editing the Feedback Script [▸ 241]*.

13.3.2.11.1.2 Commands tab

The **Commands** tab on the right contains all commands for observables, actions and editor tools.

Parameter	Description
Available Observables	Observables are conditions or parameters that can be determined and observed during the course of the experiment. Select observables, actions and tools by clicking on the black triangle at the right-hand edge and dragging the desired action from the list to the desired input area. The following observables are available:
- Analysis	Analysis observables contain measurement parameters from image analyses that have been previously defined, e.g in the Image Analysis Wizard. Only those variables that are specified through the selection of the measurement program appear in the list.
- Experiment	Experiment observables relate to the experiment currently running and display the current time point or the current Z-stack plane, for example.
- Hardware	Hardware observables include, for example, the status of connected devices, such as trigger devices, or incubators.
- Environment	Environment observables describe the system environment, such as the time or available memory space on the computer.
Available Actions	Actions are possible actions and reactions that can be performed during the experiment. These can vary greatly and include, for example, changing microscope hardware, changing camera parameters, generating notifications or audible alerts, calling up other programs or canceling the experiment.

Parameter	Description
- Experiment Actions	Commands that can be used to modify a running experiment on-the-fly. It also includes modifying hardware parameters which are typically part of an acquisition experiment, like exposure times or light source intensities.
- Hardware Actions	Hardware Actions are modifications of a running experiment concerning the incubation, XYZ positions and the Digital IO ports.
- Extra Actions	Commands that can be used to log any kind of data into a text file or to start an external application outside ZEN at any time during a running experiment.
Editor Tools	The Editor tools include sample scripts, allow to play sounds or write debugging outputs.
- Examples and Templates	Contains a small collection of script samples to illustrate some basic ideas of the feedback script. You can adopt the sample scripts by double-clicking. After receiving a prompt, the current content in the script window is overwritten entirely .
Validate Script	Checks your script for errors. An information is displayed if your script is valid or not.
Information	
- IO Card Port Labels	Contains the exact naming of the available IO ports for the current system.

13.3.2.11.1.2.1 Script Commands - Observables

Info

It is crucial to understand that observables are more than just values "that can be observed". They are required to trigger a script-run of the Loop Script.

If there are no observables used or they remain unchanged, the script will not be executed.

13.3.2.11.1.2.1.1 Observables - Analysis

The available commands inside the analysis sections depend on the definition of the image analysis pipeline and the defined ROIs from the Physiology module. Therefore this is a dynamically created list that will always look different. **The list will only contain those parameters, that are defined inside the CZIAS measurement file. Such a file can be created using the image analysis wizard or an OAD macro.**

13.3.2.11.1.2.1.2 Observables - Experiment

CurrentBlockIndex	
Complete Command	**ZenService.Experiment.CurrentBlockIndex**
Input	
Output	integer

CurrentBlockIndex	
Description	Returns the current experiment block index in heterogeneous experiments.

CurrentSceneIndex	
Complete Command	**ZenService.Experiment.CurrentSceneIndex**
Input	
Output	integer
Description	Returns the index of the current position for multi-position experiments.

CurrentTileIndex	
Complete Command	**ZenService.Experiment.CurrentTileIndex**
Input	
Output	integer
Description	Returns the current tile index.

CurrentTimePointIndex	
Complete Command	**ZenService.Experiment.CurrentTimePointIndex**
Input	
Output	integer
Description	Returns the current time point index, e.g. the frame number from a time-lapse experiment.

CurrentTrackIndex	
Complete Command	**ZenService.Environment.CurrentTrackIndex**
Input	
Output	integer
Description	Returns the current track number.

CurrentZSliceIndex	
Complete Command	**ZenService.Experiment.CurrentZSliceIndex**
Input	
Output	integer
Description	Returns the current Z-Plane form the Z-stack.

ElapsedTimeInMinutes	
Complete Command	**ZenService.Experiment.ElapsedTimeInMinutes**

ElapsedTimeInMinutes	
Input	
Output	double
Description	Returns the elapsed time in minutes for the current experiment.

ElapsedTimeInSeconds	
Complete Command	**ZenService.Experiment.ElapsedTimeInSeconds**
Input	
Output	double
Description	Returns the elapsed time in seconds for the current experiment.

ImageFileName	
Complete Command	**ZenService.Experiment.ImageFileName**
Input	
Output	string
Description	Returns the name of the current experiment as a string. The String ending will be *.czi and *.czmbi for multi-block experiments, respectively.

IsExperimentPaused	
Complete Command	**ZenService.Experiment.IsExperimentPaused**
Input	
Output	boolean
Description	Returns the paused state of the current experiment, i.e. it will be True if the experiment is paused.

IsExperimentRunning	
Complete Command	**ZenService.Experiment.IsExperimentRunning**
Input	
Output	boolean
Description	Returns the running state of the current experiment, i.e. it will be True if the experiment is running.

HasChanged	
Complete Command	**ZenService.Environment.HasChanged(String observableId)**
Input	
Output	boolean

HasChanged	
Description	Returns true/false if a certain observable has/ has not changed. On simple occurrences it is possible to use the HasChanged("observableId") method. Where available the HasChanged("observableId") method takes an observable name as parameter. HasChanged("observableId") returns true whenever the specified observable has changed since last script run; otherwise false. So it might not be suitable for situations when an observable changes very often and/or when really a singular execution is desired.

13.3.2.11.1.2.1.3 Observables - Environment

CurrentDateDay	
Complete Command	**ZenService.Environment.CurrentDateDay**
Input	
Output	integer
Description	Returns the current day.

CurrentDateMonth	
Complete Command	**ZenService.Environment.CurrentDateMonth**
Input	
Output	integer
Description	Returns the current month.

CurrentDateYear	
Complete Command	**ZenService.Environment.CurrentDateYear**
Input	
Output	integer
Description	Returns the current year.

CurrentTimeHour	
Complete Command	**ZenService.Environment.CurrentTimeHour**
Input	
Output	integer
Description	Returns the current hour.

CurrentDateTimeMinute	
Complete Command	**ZenService.Environment.CurrentTimeMinute**
Input	
Output	integer

CurrentDateTimeMinute	
Description	Returns the current minute.

CurrentDateTimeSeconds	
Complete Command	**ZenService.Environment.CurrentTimeSeconds**
Input	
Output	integer
Description	Returns the current second.

CurrentDateTimeMilliseconds	
Complete Command	**ZenService.Environment.CurrentTimeMilliseconds**
Input	
Output	integer
Description	Returns the current millisecond.

FreeDiskSpaceInMBytes	
Complete Command	**ZenService.Environment.FreeDiskSpaceInMBytes**
Input	
Output	double
Description	Returns the free disk space on the hard disk where the experiment data will be saved.

HasChanged	
Complete Command	**ZenService.Environment.HasChanged(String observableId)**
Input	
Output	boolean
Description	Returns whether a certain observable has changed. On simple occurrences it is possible to use the HasChanged("observableId") method. Where available the HasChanged("observableId") method takes an observable name as parameter. HasChanged("observableId") returns true whenever the specified observable has changed since last script run; otherwise false. So it might not be suitable for situations when an observable changes very often and/or when really a singular execution is desired.

13.3.2.11.1.2.1.4 Observables - Hardware

IncubationAirHeaterIsEnabled	
Complete Command	**ZenService.Hardware.IncubationAirHeaterIsEnabled**
Input	

IncubationAirHeaterIsEnabled	
Output	boolean
Description	Returns the state of the incubator.

IncubationAirHeaterTemperature	
Complete Command	**ZenService.Hardware.IncubationAirHeaterTemperature**
Input	
Output	double
Description	Returns the current temperature setpoint.

IncubationChannelXIsEnabled (X=1-4)	
Complete Command	**ZenService.Hardware.IncubationChannelXIsEnabled**
Input	
Output	boolean
Description	Returns the state for a specific incubation channel.

IncubationChannelXTemperature (X=1-4)	
Complete Command	**ZenService.Hardware.IncubationChannelXTemperature**
Input	
Output	double
Description	Returns the temperature for a specific channel.

IncubationCO2Concentration	
Complete Command	**ZenService.Hardware.IncubationCO2Concentration**
Input	
Output	Double
Description	Returns the current CO2 concentration setpoint.

IncubationCO2IsEnabled	
Complete Command	**ZenService.Hardware.IncubationCO2IsEnabled**
Input	
Output	Boolean
Description	Returns the state of the CO2 incubation system.

IncubationO2Concentration	
Complete Command	**ZenService.Hardware.IncubationO2Concentration**
Input	

IncubationO2Concentration	
Output	Double
Description	Returns the current O2 concentration setpoint.

IncubationO2IsEnabled	
Complete Command	**ZenService.Hardware.IncubationO2IsEnabled**
Input	
Output	Boolean
Description	Returns the state of the O2 incubation system.

IncubationCoolingChannelIsEnabled	
Complete Command	**ZenService.Hardware.IncubationCoolingChannelIsEnabled**
Input	
Output	Boolean
Description	Returns the state of the cooling system.

IncubationCoolingChannelTemperature	
Complete Command	**ZenService.Hardware.IncubationCoolingChannelTemperature**
Input	
Output	Double
Description	Returns the current cooling temperature.

IncubationHumidityIsEnabled	
Complete Command	**ZenService.Hardware.IncubationHumidityIsEnabled**
Input	
Output	Boolean
Description	Returns the state of the humidifier.

IncubationHumidityValue	
Complete Command	**ZenService.Hardware.IncubationHumidityValue**
Input	
Output	Double
Description	Returns the current humidity.

TriggerDigitalInX (X = ...)	
The range of values for X depends on the IOcard configuration.	
Complete Command	**ZenService.Hardware.TriggerDigitalInX**

TriggerDigitalInX (X = ...)	
Input	
Output	Boolean
Description	Returns the state of a specific TriggerDigitalIn port.

TriggerDigitalOutX (X = ...)	
The range of values for X depends on the IOcard configuration.	
Complete Command	**ZenService.Hardware.TriggerDigitalOutX**
Input	
Output	Boolean
Description	Returns the state of a specific TriggerDigitalOut port.

TriggerDigitalOutRLShutter	
Complete Command	**ZenService.Hardware.TriggerDigitalRLShutter**
Input	
Output	Boolean
Description	Returns the state of the reflected light shutter, where True = Open and False = Closed.

TriggerDigitalOutTLShutter	
Complete Command	**ZenService.Hardware.TriggerDigitalOutTLShutter**
Input	
Output	Boolean
Description	Returns the state of the transmitted light shutter, where True = Open and False = Closed.

13.3.2.11.1.2.2 Script Commands - Available Actions

13.3.2.11.1.2.2.1 Actions - Experiment

Depending on the present hardware the shown options might vary, e.g. it is only possible to set the laser intensity, if there is a laser engine configured.

ContinueExperiment	
Complete Command	**ZenService.Actions.ContinueExperiment()**
Input	
Output	
Description	This will continue the current experiment in case it was paused.

JumpToBlock	
Complete Command	**ZenService.Actions.JumpToBlock(int newBlockIndex)**
Input	integer newBlockIndex
Output	
Description	This will jump to the specified experiment block inside a heterogeneous experiment.

JumpToContainer	
Complete Command	**ZenService.Actions.JumpToContainer(string containerId)**
Input	string containerId
Output	
Description	This will jump to the specified container of the sample carrier.

JumpToNextBlock	
Complete Command	**ZenService.Actions.JumpToNextBlock()**
Input	
Output	
Description	This will jump to next acquisition block defined inside the Experiment Designer tool

JumpToNextContainer	
Complete Command	**ZenService.Actions.JumpToNextContainer()**
Input	
Output	
Description	This will jump to next container inside the currently used sample carrier.

JumpToNextRegion	
Complete Command	**ZenService.Actions.JumpToNextRegion()**
Input	
Output	
Description	This will jump to next defined region.

JumpToPreviousBlock	
Complete Command	**ZenService.Actions.JumpToPreviousBlock()**
Input	
Output	

JumpToPreviousBlock	
Description	This will jump to the previous acquisition block inside the experiment designer tool.

MoveTileRegion (1)	
Complete Command	**ZenService.Actions.MoveTileRegion(int regionIndex, double x, double y)**
Input	integer regionIndex, double x [nm], double y [nm]
Output	
Description	Updates the specified tile region inside the tile region list with the given X and Y coordinates.

MoveTileRegion (2)	
Complete Command	**ZenService.Actions.MoveTileRegion(int regionIndex, double x, double y, double z)**
Input	integer regionIndex, double x [nm], double y [nm], double z [nm]
Output	
Description	Updates the specified tile region inside the tile region list with the given X, Y and Z stage coordinates.

MoveTileRegionByOffset	
Complete Command	**ZenService.Actions.MoveTileRegionByOffset(int regionIndex, double offsetX, double offsetY, double offsetZ)**
Input	integer regionIndex, double offsetX [nm], double offsetY [nm], double offsetZ [nm]
Output	
Description	Updates the specified tile region inside the tile region list with the given offset in X, Y and Z.

PauseExperiment	
Complete Command	**ZenService.Actions.PauseExperiment()**
Input	
Output	
Description	This will pause the current running experiment.

ReadLEDIntensity	
Complete Command	**ZenService.Actions.ReadLEDIntensity(int trackindex, double wavelength)**
Input	integer trackindex, double wavelength [nm]
Output	double intensity

ReadLEDIntensity	
Description	This will read the intensity for the specified track and LED.

ReadTLHalogenLampIntensity	
Complete Command	**ZenService.Actions.ReadTLHalogenLampIntensity(int trackindex)**
Input	integer trackindex
Output	double intensity
Description	This will read the intensity for the specified track and LED.

SetExposureTime (1)	
Complete Command	**ZenService.Actions.SetExposureTime(int channelindex, double exposure)**
Input	integer channelindex, double exposure [ms]
Output	
Description	This will set the exposure time of the camera for the specified channel.

SetExposureTime (2)	
Complete Command	**ZenService.Actions.SetExposureTime(int trackindex, int channelindex, double exposure)**
Input	integer trackindex, integer channelindex, double exposure [ms]
Output	
Description	This will set the exposure time of the camera for the specified track and channel.

SetLEDIntensity	
Complete Command	**ZenService.Actions.SetLEDIntensity(int trackindex, double wavelength, double intensity)**
Input	integer trackindex, double wavelength [nm]
Output	
Description	This will set the intensity for the specified track and channel.

SetLEDIsEnabled	
Complete Command	**ZenService.Actions.SetLEDIsEnabled(int trackindex, double wavelength, bool isEnabled)**
Input	integer trackindex, double wavelength [nm], boolean is Enabled
Output	
Description	This will enable the specified LED for the specified track. The predefined intensity value will be used.

SetMarkerString	
Complete Command	**ZenService.Actions.SetMarkerString(string markerText)**
Input	string markerText
Output	
Description	This will insert a marker at the current time point.

SetTimeSeriesInterval (1)	
Complete Command	**ZenService.Actions.SetTimeSeriesInterval(double interval, TimeUnit unit)**
Input	double interval, TimeUnit unit (e.g. TimeUnit.ms)
Output	
Description	This will set the interval of a time series to the specified value.

SetTimeSeriesInterval (2)	
Complete Command	**ZenService.Actions.SetTimeSeriesInterval(double interval)**
Input	double interval [ms]
Output	
Description	This will set the interval of a time series to the specified value in [ms].

SetTLHalogenLampIntensity	
Complete Command	**ZenService.Actions.SetTLHalogenLampIntensity(int trackindex, double intensity)**
Input	integer trackindex, double intensity
Output	
Description	This will set the intensity of the TL Halogen lamp for the specified track.

StopExperiment	
Complete Command	**ZenService.Actions.StopExperiment()**
Input	
Output	
Description	This will stop the current running experiment (similar to pressing the **Stop** button).

UpdateZStackCenterPosition	
Complete Command	**ZenService.Actions.UpdateZStackCenterPosition(double centerPosition)**
Input	double center position [nm]

UpdateZStackCenterPosition	
Output	
Description	Updates the center position of the defined Z-stack with the specified center position.

UpdateZStackCenterPositionByOffset	
Complete Command	**ZenService.Actions.UpdateZStackCenterPositionByOffset(double centerPositionOffset)**
Input	double center position offset [nm]
Output	
Description	Moves the center position of the defined Z-stack by the specified offset.

13.3.2.11.1.2.2.2 Actions - Experiment - LSM

LSM - ReadAnalogInTriggerState	
Complete Command	**ZenServiceLSM.Actions.ReadAnalogInTriggerState(int port)**
Input	integer trigger port
Output	
Description	Returns the state of specified AnalogInTriggerIn port.

LSM - ReadAnalogOutTriggerState	
Complete Command	**ZenServiceLSM.Actions.ReadAnalogOutTriggerState(int port)**
Input	integer trigger port
Output	
Description	Returns the state of specified AnalogInTriggerIn port.

LSM - ReadDigitalGain	
Complete Command	**ZenServiceLSM.Actions.ReadDigitalGain(int trackIndex, int channelIndex)**
Input	integer trackindex, integer channelIndex
Output	double digitalGain
Description	Returns the value for the DigitalGain for the specified track/channel.

LSM - ReadLaserIntensity	
Complete Command	**ZenServiceLSM.Actions.ReadLaserIntensity(int trackIndex, int lineWavelength)**
Input	integer trackIndex, integer lineWavelength [nm]
Output	double intensity

LSM - ReadLaserIntensity	
Description	Returns the laser intensity [%] for the specified laser wavelength.

LSM - ReadMasterGain	
Complete Command	**ZenServiceLSM.Actions.ReadMasterGain(int trackIndex, int detectorIndex)**
Input	integer trackIndex, integer detectorIndex
Output	double masterGain
Description	Returns the value for the masterGain for the specified track/dectector combination.

LSM - ReadPinholeDiameter	
Complete Command	**ZenServiceLSM.Actions.ReadPinholeDiameter(int trackIndex)**
Input	integer trackIndex
Output	double pinholeDiameter [micron]
Description	Returns the value for the pinhole in [micron] for the specified track.

LSM - ReadScanDirection	
Complete Command	**ZenServiceLSM.Actions.ReadScanDirection()**
Input	
Output	ScanDirections direction
Description	Returns the current scan direction.

LSM - ReadScanSpeed	
Complete Command	**ZenServiceLSM.Actions.ReadScanSpeed()**
Input	
Output	int speed
Description	Returns the current scan speed.

LSM - ReadTLLEDIntensity	
Complete Command	**ZenServiceLSM.Actions.ReadTLLEDIntensity(int trackIndex)**
Input	integer trackIndex
Output	double intensity [%]
Description	Returns the intensity value [%] for the TL-LED.

LSM - SetAnalogOutTriggerState	
Complete Command	**ZenServiceLSM.Actions.SetAnalogOutTriggerState(int port, double state)**

LSM - SetAnalogOutTriggerState	
Input	integer port, double state
Output	
Description	Sets the voltage value for the AnalogOutTrigger port.

LSM - SetDigitalGain	
Complete Command	**ZenServiceLSM.Actions.SetDigitalGain(int trackIndex, int channelIndex, double digitalGain)**
Input	integer trackIndex, integer channelIndex, double digitalGain
Output	
Description	Sets the value for the DigitalGain for the specified track/channel.

LSM - SetLaserEnabled	
Complete Command	**ZenServiceLSM.Actions.SetLaserEnabled(int trackIndex, int lineWavelength, bool isEnabled)**
Input	integer trackIndex, integer lineWavelength, bool isEnabled
Output	
Description	Sets the state for the specified laser inside the specified track.

LSM - SetLaserIntensity	
Complete Command	**ZenServiceLSM.Actions.SetLaserIntensity(int trackIndex, int lineWavelength, double intensity)**
Input	integer trackIndex, integer lineWavelength [nm], double intensity [%]
Output	
Description	Sets the laser intensity for the specified laser inside the specified track.

LSM - SetMasterGain	
Complete Command	**ZenServiceLSM.Actions.SetMasterGain(int trackIndex, int detectorIndex, double masterGain)**
Input	integer trackIndex, integer detectorIndex, double masterGain
Output	
Description	Sets the value for the MasterGain for the specified track/detector.

LSM - SetPinholeDiameter	
Complete Command	**ZenServiceLSM.Actions.SetPinholeDiameter(int trackIndex, double pinholeDiameter)**
Input	integer trackIndex, double pinholeDiameter [nm]
Output	

LSM - SetPinholeDiameter	
Description	Adjusts the pinhole for the specified track.

LSM - SetScanDirection	
Complete Command	**ZenServiceLSM.Actions.SetScanDirection(ScanDirections direction)**
Input	ScanDirections direction
Output	
Description	Adjust the ScanDirection to the specified value.

LSM - SetScanSpeed	
Complete Command	**ZenServiceLSM.Actions.SetScanSpeed(int speed)**
Input	integer speed
Output	
Description	Adjusts the ScanSpeed to the specified value.

LSM - SetTLLEDIntensity	
Complete Command	**ZenServiceLSM.Actions.SetTLLEDIntensity(int trackIndex, double intensity)**
Input	integer trackIndex, double intensity [%]
Output	
Description	Adjusts the intensity of the TL-LED to the specified value.

13.3.2.11.1.2.2.3 Actions - Hardware

ExecuteHardwareSetting	
Complete Command	**ZenService.HardwareActions.ExecuteHardwareSetting(string settingNameInExperimentSettingsPool)**
Input	string settingNameInExperimentSettingsPool
Output	
Description	Loads a specific experiment setting from the list of experiment settings. **The settings will be overwritten within the next experiment loop, if the settings are included in the regular experiment settings.**

ExecuteHardwareSettingFromFile	
Complete Command	**ZenService.HardwareActions.ExecuteHardwareSettingFromFile(string hardwareSettingFilePath)**
Input	integer string hardwareSettingFilePath
Output	

ExecuteHardwareSettingFromFile	
Description	Loads a specific experiment setting from a file directly. **The settings will be overwritten within the next experiment loop, if the settings are included in the regular experiment settings.**

PulseTriggerDigitalOut	
Complete Command	**ZenService.HardwareActions.PulseTriggerDigitalOut(string port-Label, double duration)**
Input	string portLabel, double duration
Output	
Description	Produces a TLL pulse with a specified duration at the selected trigger port.

PulseTriggerDigitalOutX (X=7-8)	
Complete Command	**ZenService.HardwareActions.PulseTriggerDigitalOutX(double duration)**
Input	double duration
Output	
Description	Produces a TLL pulse with a specified duration at trigger port X.

ReadFocusPosition	
Complete Command	**ZenService.HardwareActions.ReadFocusPosition(double Position- InMicrometer)**
Input	double PositionInMicrometer
Output	
Description	Get the current value of the Z-drive. This function was placed under the category **HardwareActions**, and not under **Observables**, since they trigger the execution of the main loop of the script automatically when changing.

ReadStagePositionX	
Complete Command	**ZenService.HardwareActions.ReadFocusPosition(double Position- InMicrometer)**
Input	double PositionInMicrometer
Output	
Description	Get the current value of the stage X-axis. This function was placed under the category **HardwareActions**, and not under **Observables**, since they trigger the execution of the main loop of the script automatically when changing.

ReadStagePositionY	
Complete Command	**ZenService.HardwareActions.ReadFocusPosition(double Posi- tion- InMicrometer)**
Input	double PositionInMicrometer
Output	
Description	Get the current value of the stage Y-axis. This function was placed under the category **HardwareActions**, and not under **Observables**, since they trigger the execution of the main loop of the script automatically when changing.

SetFocusPosition	
Complete Command	**ZenService.HardwareActions.SetFocusPosition(double PositionInMicrometer)**
Input	double PositionInMicrometer
Output	
Description	Sets the focus drive to the specified position. Currently the piezo drive is not accessible from the Experiment Feedback script.

SetIncubationAirHeaterIsEnabled	
Complete Command	**ZenService.HardwareActions.SetIncubationAirHeaterIsEnabled(bool IsEnabled)**
Input	bool IsEnabled
Output	
Description	Enables the AirHeater of the incubation system.

SetIncubationAirHeaterTemperature	
Complete Command	**ZenService.HardwareActions.SetIncubationAirHeaterTemperature(double temperature)**
Input	double temperature
Output	
Description	This will set the temperature of the AirHeater to the specified temperature.

SetIncubationChannelIsEnabled	
Complete Command	**ZenService.HardwareActions.SetIncubationChannelIsEnabled(int channel, bool isEnabled)**
Input	integer channel, boolean isEnabled
Output	double intensity
Description	This will enable the specified channel.

SetIncubationChannelTemperature	
Complete Command	**ZenService.HardwareActions.SetIncubationChannelTemperature(int channel, double temperature)**
Input	integer channel, double temperature
Output	
Description	Sets the temperature of the selected channel to the specified value.

SetIncubationCO2Concentration	
Complete Command	**ZenService.HardwareActions.SetIncubationCO2Concentration(double concentration)**
Input	double concentration
Output	
Description	Sets the CO2 concentration to the specified value.

SetIncubationCO2IsEnabled	
Complete Command	**ZenService.HardwareActions.SetIncubationCO2IsEnabled(bool isEnabled)**
Input	boolean isEnabled
Output	
Description	Enables the CO2 incubation.

SetIncubationO2Concentration	
Complete Command	**ZenService.HardwareActions.SetIncubationO2Concentration(double concentration)**
Input	double concentration
Output	
Description	Sets the O2 concentration to the specified value.

SetIncubationO2IsEnabled	
Complete Command	**ZenService.HardwareActions.SetIncubationO2IsEnabled(bool isEnabled)**
Input	boolean isEnabled
Output	
Description	Enables the O2 incubation.

SetStagePosition	
Complete Command	**ZenService.HardwareActions.SetStagePosition(double positionXInMicrometer, double positionYInMicrometer)**
Input	double positionXInMicrometer, positionYInMicrometer

SetStagePosition	
Output	
Description	Sets the stage XY position to the specified value.

SetStagePositionX	
Complete Command	**ZenService.HardwareActions.SetStagePosition(double positionXInMicrometer)**
Input	double positionXInMicrometer
Output	
Description	Sets the stage X position to the specified value.

SetStagePositionY	
Complete Command	**ZenService.HardwareActions.SetStagePosition(double positionYInMicrometer)**
Input	double positionYInMicrometer
Output	
Description	Sets the stage Y position to the specified value.

SetTriggerDigitalOut	
Complete Command	**ZenService.HardwareActions.SetTriggerDigitalOut(string portLabel, bool isSet)**
Input	string portLabel, boolean isSet
Output	
Description	Sets the selected TriggerDigitalOut port to the specified value.

SetTriggerDigitalOut7	
Complete Command	**ZenService.HardwareActions.SetTriggerDigitalOut7(bool isSet)**
Input	boolean isSet
Output	
Description	This will set TriggerDigitalOut7 port to the specified value.

SetTriggerDigitalOut8	
Complete Command	**ZenService.HardwareActions.SetTriggerDigitalOut8(bool isSet)**
Input	boolean isSet
Output	
Description	Sets the selected TriggerDigitalOut8 port to the specified value.

SetTriggerDigitalOutRLShutter	
Complete Command	**ZenService.HardwareActions.SetTriggerDigitalOutRLShut-ter(bool isSet)**
Input	boolean isSet
Output	
Description	Sets the TriggerDigitalOutRLShutter port to the specified value.

SetTriggerDigitalOutTLShutter	
Complete Command	**ZenService.HardwareActions.SetTriggerDigitalOutTLShut-ter8(bool isSet)**
Input	boolean isSet
Output	
Description	Sets the selected TriggerDigitalOutTKShutter port to the specified value.

13.3.2.11.1.2.2.4 Actions - Extra

AppendLogLineString (1)	
Complete Command	**ZenService.Xtra.System.AppendLogLineString(string logMes-sage)**
Input	string logMessage
Output	string (contains the file path of the data log file)
Description	Creates a text file (ExperimentName_Log.txt) inside the folder save folder/temp and the file path to the log file is returned. Numbers have to be converted to strings in order two write them into the log file. Column separators have to be specified as strings as well.

AppendLogLineString (2)	
Complete Command	**ZenService.Xtra.System.AppendLogLineString(string logMes-sage, string logFileName)**
Input	string logMessage, string logFileName
Output	string (contains the file path of the data log file)
Description	This is similar to the function AppendLogLineString (1) but allows to specify the file path where the text file will be saved.

ExecuteExternalProgram (1)	
Complete Command	**ZenService.Xtra.System.ExecuteExternalProgram(string exe-FilePath)**
Input	string exeFilePath
Output	

ExecuteExternalProgram (1)	
Description	Starts an external application (*.exe, *.py, *.mp3, ...). It may be required to add the location of the application the environmental variables, usually PATH, in order for windows to find the application. It is also possible to use the absolute file path.

ExecuteExternalProgram (2)	
Complete Command	**ZenService.Xtra.System.ExecuteExternalProgram(string exeFilePath, string arguments)**
Input	string exeFilePath, string arguments
Output	
Description	This is similar to the function above but it allows to specify arguments which can be passed to the application. An example could look like this: ZenService.Xtra.ExecuteExternalProgram("fiji-win64.exe", "-macro Open_CZI_OME_complete.ijm Experiment-123.czi")

ExecuteExternalProgramBlocking (1)	
Complete Command	**ZenService.Xtra.System.ExecuteExternalProgram(string exeFilePath, string arguments, int timeoutInMs)**
Input	string exeFilePath, string arguments, int timeoutInMs
Output	integer exitCode
Description	This advanced method will run an external program and blocks the subsequent script. When the Experiment Feedback runs in synchronous script execution it also blocks the experiment until the external application is closed. It requires a paramater called timeoutinMs, which specifies after which time period the script will continue independent from the external applications. As an output it returns a so-called ExitCode (integer), which is produced by the external application when exiting. This ExitCode has to be supported by the external application. Standard Windows programs (like Notepad, etc.) just return '0'. When using a special application or self-written applications it is possible to set a special own ExitCode.

ExecuteExternalProgramBlocking (2)	
Complete Command	**ZenService.Xtra.System.ExecuteExternalProgram(string exeFilePath, int timeoutInMs)**
Input	string exeFilePath, int timeoutInMs
Output	integer exitCode
Description	This is the same method as above but with additional arguments for the external application. The timeout parameter is still required.

PlaySound (1)	
Complete Command	**ZenService.Xtra.System.PlaySound()**
Input	

PlaySound (1)	
Output	
Description	This will play the system sound.

PlaySound (2)	
Complete Command	**ZenService.Xtra.System.PlaySound(string waveFilePath)**
Input	string waveFilePath
Output	
Description	Plays the specified wave sound file.

RunLoopScript	
Complete Command	**ZenService.Xtra.System.RunLoopScript()**
Input	
Output	
Description	This command will trigger a run of the LoopScript. It is only meant to be used when running the Experiment Feedback script in synchronous script execution.

WriteDebugOutput	
Complete Command	**ZenService.Xtra.System.WriteDebugOutput(string message)**
Input	string
Output	
Description	Writes string messages in the Output for debugging.

13.3.2.11.1.3 Debugging tab

Parameter	Description
General	
- Show Warning Popup	Allows to open warning popups.
- Show Output Messages	Opens the output messages field. Here the messages defined below are displayed during script run for debugging purposes.
Output Messages	
- Show	Displays the corresponding message in white.
- Highlight	Displays the corresponding messages with different colors.
User Defined Messages	

Parameter	Description
- Write to Output	Writes user defined messages (ZenService. Xtra.System. WriteDebugOutput (string message)).
Auto Messages	
- Observable Change	Writes a notification each time an observable changes.
- Action Called	Writes a notification each time an action is called.
- Action Executed	Writes a notification each time an action is executed.
- Action Warning	Writes action warning messages.
- Exception	Writes exception messages.
- Script Run	Writes which part of the sript (preloop, loop script, post loop script) is run.
Additional Information	
- Show Message Type	Allows to additionally display the message type.
- Show Time Stamp	Allows to additionally display the time stamp for each message.
- Follow Debug Message	Follows the output if activated.
Clear output	Clears the messages in the output field.

13.3.2.12 Timed Bleaching Tool

This tool permits setting the bleach parameters for a bleaching experiment in combination with a time series. Bleaching or photo manipulation is done in between acquisition.

Parameter	Description
Start after # of images	**Activated:** Enables you to set the number of frames that are imaged before the bleaching process.
Repeat after # of images	**Activated:** Enables you to set the number of images that will separate a repetitive bleaching procedure.
- Iterations	Defines the bleach repetitions for bleach areas. This defines the total amount of scans which are performed for bleaching the selected region during each bleaching process.
- Spot Bleach Duration	This function is only available with LSM 980. When spots are defined for bleaching enter the time for each spot to be bleached in this editing field.

Parameter	Description
Excitation of Bleach	
- Use different settings for different regions	**Activated:** For each previously drawn ROI a tab is present, in which laser line and laser power can be chosen.
All Regions	Only visible if the checkbox above is not activated. Note that for high laser power the **High Intensity Laser Range** must be set in the **Imaging Setup** or **Channels** tool (only for LSM 900). Activates the according checkbox for a laser line. Use the slider to adjust the power for bleaching/photo-manipulation.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Bleaching Settings	Here you can manage bleaching settings, e.g. save, load or delete settings on the hard disk.
Stop on intensity below	**Activated:** Enables you to bleach the sample to an intensity value calculated as % of the initial intensity within the region to bleach, during an experiment with repetitive bleaching. The intensity value is determined with every taken image. Note: This function can only be applied if one ROI for bleaching is defined. It does not work for multiple regions.
Set Different scan speed	**Activated:** Enables you to set the scan speed for bleaching can differently than the imaging scan speed. A lower speed results in a longer pixel dwell time, which increases the efficiency of bleaching.
Set different Z-Position	**Activated:** Enables you to perform the bleaching at a different z-level than currently set. Enter the difference in µm into the input field.
Spot/Full area sequential bleach	**Activated:** For Spot Bleach mode this is set as default. When activated all regions (and spots) are bleached sequentially performing all bleach iterations for each region first before moving to the next region. The scan field is limited to each bleach region.
Protect detectors during bleach	**Activated:** PMT gain is set to 0 V during the bleaching step. Note that, selecting this option the PMT gain will slow down the acquisition. Typically, small bleach ROIs will not be causing a critically high signal for the detectors. However, even when this option is not activated, the detectors will either downregulate the voltage or shut down completely in case of significant over exposure.

13.3.2.13 Interactive Bleaching Tool

This tool allows to bleach interactively during a **Continuous** scan or during a **Time Series** experiment while image acquisition is performed. The bleach region is determined by pointing the mouse onto the position in the image.

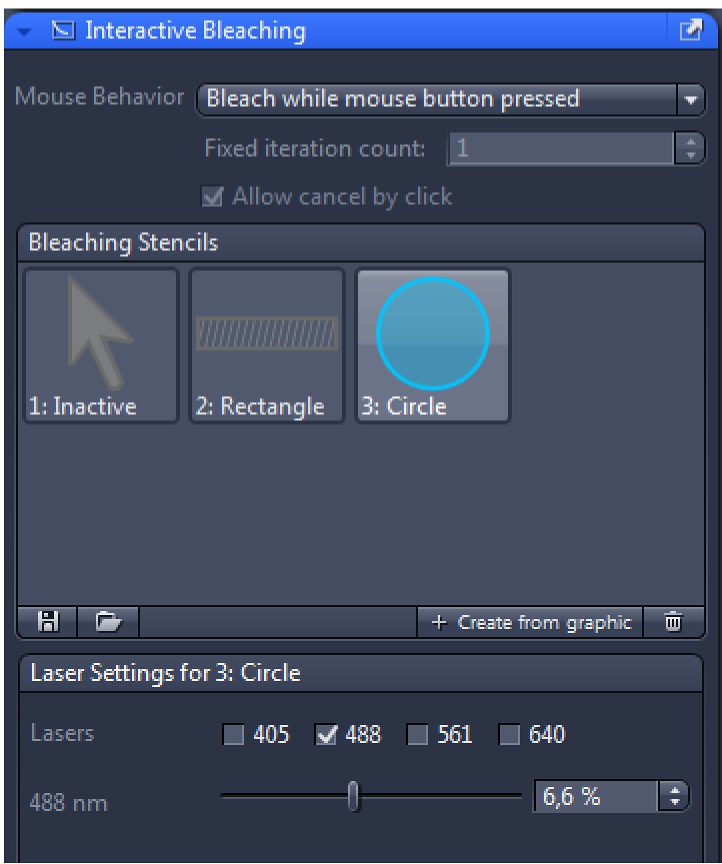


Fig. 69: Interactive Bleaching Tool

Parameter	Description
Mouse Behavior	Change the display of the selected clipping plane using the dropdown list to the right of the **Activate** checkbox. The following settings are available:
- Bleach while mouse button pressed	The bleaching process is continued while the mouse button is pressed.
- Bleach fixed number of iterations	The bleaching process is continued for a fixed number of times after the mouse is pressed. The number of iterations can be entered below.
- Fixed iteration count	Determines the number of iterations.
- Allow cancel by click	If activated, the bleaching process can be stopped before the fixed number iterations is accomplished. Simply click on the left mouse button again to stop the bleaching process.

Parameter	Description
Bleaching Stencils list	The available bleaching stencils (equivalent to ROIs for bleaching) are listed here. A stencil is displayed in its graphical form, the color displays the laser line that is assigned for bleaching to this stencil. You can add a stencil by clicking the **+ Create from graphic** button. This will import an activated graphical element from the **Graphics** tool tab. A stencil can be saved, loaded or deleted. A stencil is active, when clicked on and highlighted. To use an activated stencil for bleaching, move the mouse cursor onto the image and click on the left mouse button.
Laser Settings for section	Here you can set the laser line and laser power for the activated stencil.

13.3.2.14 Dynamics Tool

The checkbox to activate the tool is only visible if **Time Series** acquisition is active and **Tiles** is not activated.

Using the tool you can open the **Mean ROI Setup** where you can configure physiology functions for a **Time Series** experiment. Deactivate the **Dynamics** checkbox to deactivate physiology functions.
If you click on the **Mean ROI Setup** button the setup view will be visible in the **Center Screen Area**. There you will see a snapshot containing all channels of the currently active image.

13.3.2.14.1 Mean ROI Setup

The **Mean ROI Setup** is in essence a modified version of the **MeanROI** view with several minor additions. It allows experiment pre-settings to be made on Snap-shots of the cells/ specimen on which the measurements will be made. These settings include:

- image and chart layouts;
- chart axis settings;
- generation, placement and management of measurement and Background ROIs
- measurement settings

A ratio preview image is also displayed that allows an assessment of the current ratio settings on the Online Ratio tab. To update the images in the Mean ROI Setup a new Snap can be made at any time.

See also

Mean ROI Tab [▸ 861]
Layout Tab [▸ 862]
Charts Tab [▸ 863]
Online Ratio Tab [▸ 864]
Mean ROI View [▸ 859]

13.3.2.15 Auto Save Tool

Info

The Auto Save tool is not visible, if you have activated the **Panorama** checkbox in the Experiment Manager. When you execute an experiment or click on the **Snap** button, the Auto Save tool is disabled until the experiment is cancelled or finished. The tool also stays disabled when the experiment is paused. This behavior should prevent operation errors during experiments.

If the **Auto Save** checkbox in the **Experiment Manager** is activated, all images which are acquired from the Acquisition tab, are automatically stored as ***.CZI** format during acquisition.

Generally, all images are automatically written to the hard disk during acquisition. This is to prevent data loss in case of technical problems. The folder path for these files is displayed in the status bar under **Storage Folder**. The location can easily be opened by double-clicking on this field. The path can be changed in the **Tools** menu | **Options** | **Saving**. Even though these files are stored physically on the disk, they are indicated with an asterisk and you will be prompted to either rename and store them in a different place, or to delete them. They are maintained only if the software crashes. In case you want to store the files directly in a location of your choice, activate the **Auto Save** checkbox. In this case the files are not written to the temporary folder anymore.

The automatically saved images are contained in the subfolder **temp** within the currently chosen image storage path (default path is: C:\Users\<username>\Pictures\temp). When saving such temporarily saved images via the **File** menu | **Save** you will be asked to specify a document name and a storage location. If you close such an image document without saving it, it will be permanently deleted from the Temp folder in order to prevent the accumulation of unnecessary images.

Parameter	Description
Folder	Shows the directory for the images. The text box is read-only. No values can be entered or pasted by the user. This ensures that the text box contains always a valid directory To change the directory click on the ... button to select a new folder for the auto saved images.
Automatic Sub-Folder	**Activated:** Creates automatically a top level sub folder in the given directory. The sub folder name is based on the actual date, e. g. 2014-07-04.
Name	Here you can specify the image name. An index number is automatically appended to the image name.
Close CZI after acquisition	**Activated:** Closes the CZI image in the center screen area when the experiment is finished.
File Name Preview	Shows the currently chosen storage path as well as a preview of file name being used next.

13.3.2.16 Automated Image Export Tool

Info

It is not possible to automatically export images created by the **Snap** function. If you require to use the Auto Export feature for individual images, you must create a Time Series experiment with a single cycle.

If the **Automated Export** checkbox in the **Experiment Manager** is activated before an experiment is executed, the generated images will be stored in the defined directory with the given parameters and options, provided by the options under the checkbox. This option was developed for automatically exporting images with a user defined format (TIFF or JPEG).

Info

When you execute an experiment, the tool is disabled until the experiment is cancelled or finished. The tool also stays disabled when the experiment is paused. This behavior should prevent operation errors during experiments.

For technical reasons images acquired from the Acquisition tab are always auto-saved temporarily as CZI files. If the application requires images to be stored in external common file formats, it is necessary to run the export function. The Automatic Image Export facilitates this in a convenient und automatic way giving the choice of single page TIFF or JPEG file formats. It is also possible to automatically close and discard the auto-saved CZI file to streamline the acquisition workflow.

Parameter	Description
Folder	Shows the directory for the images. The text box is read-only. No values can be entered or pasted by the user. This ensures that the text box contains always a valid directory To change the directory click on the ... button. Select a new folder for the auto saved images in the dialog.
Automatic Sub-Folder	**Activated:** Creates automatically a top level sub folder in the given directory. The sub folder name is based on the actual date, e. g. 2014-07-04.
Prefix	Here you can define a prefix for the image file name and a name for the sub folder. If the text box is empty an image gets a localized default prefix (“Untitled”) and a folder gets a localized default name (“New folder”). If an image or folder with a name still exists, the new image or folder gets the same name with an increasing index in accordance to the standard Windows Explorer behavior, e. g. New Folder (1), New Folder (2).
Format	Here you select the format for the export images. Two formats are supported: ▪ **TIFF**: For the TIFF format (lossless, bigger file size) you can additionally select the **Compression** method. – None – LZW – ZIP

Parameter	Description
	▪ **JPEG**: For the JPEG format (lossy, smaller file size) you can set the **Quality** level by adjusting the slider between **Low** (lower quality, smaller images) to **High** (higher quality, bigger file size).
Original data	For **TIFF** format only: **Activated:** Generates an additional raw data TIFF image. Its bit depth depends on the original camera image (Gray 8/16 bit or RGB 24/48 bit)
Gray Scale Linear	For **JPEG** format only: **Activated:** Generates an additional raw data JPEG image. The bit depth depends on the original camera image. In case of 8 bits, the JPEG image has 8 bit gray scale or 24 RGB. In case of 16 bits, the JPEG image is reduced to the "Valid Bits" of the camera. If the camera image is a 16 bit gray scale image, the resulting JPEG image is a 8 bit gray scale image. In case of a 48 bit RGB image the JPEG image has 8 bit RGB. Both additional image types are marked with an **ORG** suffix in their file names.
Apply display curve and channel color	**Activated:** Applies the display curve and channel color to the JPEG or TIFF image
Use channel names	**Activated:** The name of the resulting image contains the name of the defined channel.
Add XML Metadata	**Activated:** Saves an additional xml file with image meta data. Its name has the following nomenclature: Prefix_Metadata(image format).xml → Test_Metadata(tif).xml If more than one xml file with the same name exists the file gets an index, e.g. Test-02_Metadata(tif).xml.
Close CZI image after acquisition	**Activated:** Closes the CZI image in the center screen area when the experiment is finished. **NOTICE! If Auto Save is not activated, this will lead to the loss of the original .czi file for the experiment.**
Dimension/Sub-directory	If you check one of the Channels, Time Series, Z-Stack or Scenes checkboxes, an additional sub-directory will be created if the corresponding dimension exists in the experiment block. The sub-directory will be created in the same image dimension order as the CZI image created, e.g. T-C-Z. The top level folder within the "Dimension" folders is always the B ("Block") folder [new]. Each sub-directory gets a letter that represents its image dimension (T for time series, C for channel, etc.) and an index, if more than one dimension of the same type exists (T=0, T=1).

13.3.2.17 Automation Tool

With this tool you can select OAD macros which will be executed before or/and after an experiment. The default folder for macros is **User/Documents/Carl Zeiss/ZEN/Documents/Macros**. Any other folder is selectable.

Parameter	Description
Run OAD macro before experiment execution	If activated, the selected macro is executed before the experiment starts. If you click on the **...** button you can select a macro from the file system.
Run OAD macro after experiment execution	If activated, the selected macro is executed after the experiment. If you click on the **...** button you can select a macro from the file system.

13.3.3 Tools on Processing Tab

13.3.3.1 Image Parameters - Input Tool

Parameter	Description
Input image	Input image with file name and path to the target folder.
Set Input Automatically	**Activated**: The currently active image is automatically loaded as input image. This can be used to load the output image of the first image processing step as input for the next image processing step.
Apply to preview, only	After image processing, the output image document is active.
Switch to Output	**Activated**: Applies the selected Image Processing Function only to the area that is selected in the Output Tool via **Preview**.
Remain at current view	**Activated**: The currently active image document will remain active after the image processing step.

13.3.3.2 Image Parameters - Output Tool

Parameter	Description
Output image	Output image with file name and path to the target folder.
Custom Output Name	Enter a valid output name.
Overwrite	**Activated:** Overwrites an existing file.
Create New Output	**Activated:** Saves the image file with a new name.
Save outputs	**Activated:**Saves the image automatically.
Naming	Opens a dialog to define conventions for the image file name.
Preview	Displays a preview window in your image. In this window you can see a calculated preview of what the result will look like with the current parameter settings.

See also

Image Processing Workflow [90]

13.3.4 Tools on Analysis Tab

13.3.4.1 Interactive Measurement Tool

Feature Set section

Parameter	Description
Feature Set	Here you select and load previously saved feature definitions/feature sets. If you have made changes to a feature definition, the name of the feature selection is marked with an asterisk (*). If you close the application without saving a changed ("asterisked") feature selection, you will be asked whether you want to save the changes.
Options	Opens the Options shortcut menu.
Define	Opens the Feature Selection Dialog.

Feature Subset section

Only visible if the **Show All** mode is activated.

Parameter	Description
Feature Subset	Here you can select and load previously saved definitions of subsets. If you have made changes to a subset definition, the name of the feature subset is marked with an asterisk (*). If you close the application without saving a changed ("asterisked") feature subset, you will be asked whether you want to save the changes.
Options	Opens the Options shortcut menu.
Define	Opens the *Feature Subset Definition Dialog [▸ 772]*.

Measurement Sequence section

Only visible if the **Show All** mode is activated.

Parameter	Description
Measurement Sequence	Here you can select and load previously saved measurement procedures. If you have made changes to a measurement procedure, the name of the measurement procedure is marked with an asterisk (*). If you close the application without saving a changed ("asterisked") measurement procedure, you will be asked whether you want to save the changes.
Options	Opens the Options shortcut menu.
Define	Opens the Interactive Measurement Sequence Definition Dialog.

Parameter	Description
Run	Starts the selected Measurement Sequence in the Interactive Measurement Sequence Execution Dialog.

Parameter	Description
Create Measurement Table	Creates a measurement data table. This contains the measurement data from the **Measure** view of the current image.

13.3.4.1.1 Feature Selection Dialog

Here you can specify which features are measured with the available graphic elements. This selection is adopted into the current feature definition. The feature definition is then marked ("asterisked") as having been changed.

Available Elements section

In this section you can specify for each available graphic element which features you want to be measured. The graphic elements are ordered by type. The following types are available for selection:

Element Type	Description
Regions (2D)	Here you will find all the graphic elements that define a closed region.
Single Distances	Here you will find all the graphic elements with which you can measure a single distance.
Multiple Distances	Here you will find all the graphic elements with which you can measure several distances at once.
Angle	Here you will find the graphic elements with which you can measure an angle.
Point	Here you will find the graphic elements with which you can perform measurements at a pixel.
Events	Here you will find the graphic elements with which you can count various events in an image.

Selected Features section

The features that you have selected for each individual graphic element are listed in this section.

Activate the **Display** checkbox for each feature to display the value of the measured feature in the graphics plane of the image.

Parameter	Description
Delete	Deletes the selected feature.
Upwards	Moves the selected feature one position higher.

Parameter	Description
Downwards	Moves the selected feature one position lower.

Features section

All the features that you can measure with the graphic element activated in the **Available Elements** section are listed in this section.

Parameter	Description
Search Features	Here you can enter parts of the name of the feature that you are looking for. The features in which the entered character string occurs are listed. Select a type of feature according to which you want the features to be filtered from the dropdown list.
- All	All features are listed.
- Geometric Features	All geometric features are listed.
- Intensity Features	All features that analyze intensity values are listed.
- Image Features	All features that contain meta information about the measured image are listed.
- Position Features	All features that describe the position are listed.
- Geometric Features Unscaled	All features that describe unscaled geometric features.
- Position Features Unscaled	All features that describe unscaled positions are listed.
- Polygon-based Features	All features polygon-based features are listed.
- Statistical Features	All features that can be used for plotting in a heatmap (i.e. that provide statistical values suitable for heatmap plotting) are listed.

Parameter	Description
Add	Click on the button to select a feature for the measurement.

13.3.4.1.2 Feature Subset Definition Dialog

Here you can specify which features are available in the Feature Selection Dialog. These features are adopted into the current subset definition. The subset definition is then marked ("asterisked") as having been changed.

Features section

All the features that you can measure with the graphic element activated in the **Available Elements** section are listed in this section.

Parameter	Description
Search Features	Here you can enter parts of the name of the feature that you are looking for. The features in which the entered character string occurs are listed. Select a type of feature according to which you want the features to be filtered from the dropdown list.
- All	All features are listed.
- Geometric Features	All geometric features are listed.
- Intensity Features	All features that analyze intensity values are listed.
- Image Features	All features that contain meta information about the measured image are listed.
- Position Features	All features that describe the position are listed.
- Geometric Features Unscaled	All features that describe unscaled geometric features.
- Position Features Unscaled	All features that describe unscaled positions are listed.
- Polygon-based Features	All features polygon-based features are listed.
- Statistical Features	All features that can be used for plotting in a heatmap (i.e. that provide statistical values suitable for heatmap plotting) are listed.

Parameter	Description
Feature	There is a checkbox in front of the name of each of the listed features. Activate the checkbox in front of the features that you want to be offered in the **Feature Selection** dialog.
Shortcut menu	Right-click in the Features section to open the shortcut menu.
- Select All Features	Activates all checkboxes.
- Deselect All Features	Deactivates all checkboxes.

13.3.4.1.3 Interactive Measurement Sequence Definition Dialog

Here you can define an interactive measurement procedure. You can specify the order in which you want the individual graphic elements to be drawn in and which measurement parameters you want to have calculated for them. The definition is adopted into the measurement procedure currently selected. The measurement procedure is then marked ("asterisked") as having been changed.

Available Elements section

In this section you can specify for each available graphic element which features you want to be measured. The graphic elements are ordered by type. The following types are available for selection:

Element Type	Description
Regions (2D)	Here you will find all the graphic elements that define a closed region.
Single Distances	Here you will find all the graphic elements with which you can measure a single distance.
Multiple Distances	Here you will find all the graphic elements with which you can measure several distances at once.
Angle	Here you will find the graphic elements with which you can measure an angle.
Point	Here you will find the graphic elements with which you can perform measurements at a pixel.
Events	Here you will find the graphic elements with which you can count various events in an image.

Double-click on a available element to select it and adopt it into the **Selected Elements Sequence** list.

Selected Elements Sequence section

This list displays the selected graphic elements in the order in which they will be drawn in during the measurement, from top to bottom. To display the value of the measured feature in the image's graphics plane, activate the corresponding checkbox of the graphic elements.

Parameter	Description
Delete	Deletes the selected feature.
Upwards	Moves the selected feature one position higher.
Downwards	Moves the selected feature one position lower.

Features section

All the features that you can measure with the graphic element activated in the **Available Elements** section are listed in this section.

Parameter	Description
Search Features	Here you can enter parts of the name of the feature that you are looking for. The features in which the entered character string occurs are listed. Select a type of feature according to which you want the features to be filtered from the dropdown list.
- All	All features are listed.

Parameter	Description
- Geometric Features	All geometric features are listed.
- Intensity Features	All features that analyze intensity values are listed.
- Image Features	All features that contain meta information about the measured image are listed.
- Position Features	All features that describe the position are listed.
- Geometric Features Unscaled	All features that describe unscaled geometric features.
- Position Features Unscaled	All features that describe unscaled positions are listed.
- Polygon-based Features	All features polygon-based features are listed.
- Statistical Features	All features that can be used for plotting in a heatmap (i.e. that provide statistical values suitable for heatmap plotting) are listed.

Parameter	Description
Add	Click on the button to select a feature for the measurement.

13.3.4.2 Image Analysis Tool

Note that this tool is available only when you have licensed the **Image Analysis** module.

Parameter	Description
Setting	Select and load previously saved analysis programs here.
Options	Opens the options menu.
– New	Creates a new analysis setting. Enter a name for the setting.
– New from Template	Creates a new setting based on an existing setting. The template setting will not be modified.
– Create new from analyzed image	Reads out czias settings from a previously analyzed image and creates a new setting from that.
– Rename	Enables you to enter a new name for the setting.
– Save	Saves a modified setting under the current name. An asterisk indicates the modified state.
– Save As	Saves the current setting under a new name. Enter a name for the setting.
– Import	Imports an existing setting.

Parameter	Description
– Delete	Deletes the current setting.
Edit	Opens the **Segmentation Method Selection** dialog. For more information, see *Creating a new image analysis setting [▸ 369]*.

Parameter	Description
Setup Image Analysis	Opens the *Image Analysis Wizard [▸ 777]* to define a new analysis program or to change an existing program.
Analyze Interactively	Runs the selected analysis program with all the interactive steps.
Analyze	Runs the selected analysis program without interruption.

Info

Steps that you have not marked as interactive in the **Image Analysis Wizard** are run with the values set in the analysis program. The program does **not** stop to allow you to change these interactively.

13.3.4.2.1 Segmentation Method

Parameter	Description
Method	
- Segment region classes independently	For each defined region class the specified image channel will be segmented using the class segmenter which can be defined in step **Automatic Segmentation** of the image analysis wizard.
- ZOI (Zones of Influence)	Constructs a zone of influence (ZOIs) and a ring around each primary object.
- Segment binary images	For each region class, the selected image channel will be segmented by the binary class segmenter.
- Whole image	Uses the whole image as a region.
- Interactive segmentation	Uses only the interactive segmentation step. For each region class, the regions must be drawn manually.
Wizard Steps	
- Classes	In this step you can define the classes into which the measured objects in the image are divided.
- Frame	In this step you can define one or more measurement frames.
- Automatic Segmentation	In this step you can choose the segmentation method to be applied and set parameters for the segmentation of the objects that you want to measure.
- Region Filter	In this step you can define the conditions under which you want an object to be measured.

Parameter	Description
- Interactive Segmentation	In this step you can post-process the segmented objects interactively.
- Features	In this step you select the measurement features.
- Results Preview	In this step you will see the preview of the measurement result.

13.3.4.2.2 Image Analysis Wizard

Note that this wizard is available only when you have licensed the **Image Analysis** module.

13.3.4.2.2.1 Classes

In this step you can define the classes into which the measured objects in the image are divided.

Parameter	Description
Interactive	**Activated**: The class definition can be changed interactively while the measurement program is running in **Analyze Interactively** mode.
Classes	The defined classes are listed here. If you create a new measurement program, a predefined set of classes is created automatically. Each class consists of two entries. The first entry concerns all the objects belonging to the class. The second entry represents an individual object.
Add Class	Adds a new individual class to the list on the Base-level.
Add Subclass	Adds a new subclass to the selected class.
Remove Class	Deletes the selected class from the list.
Name	Here you can enter a name for the selected class in the **Classes** list.
Channel	If you create a measurement program for a multichannel image, in this selection field you need to select the channel for the selected class in the **Classes** list. This channel is used for image segmentation.
Color	Here you can select a color to mark the objects of a class.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard.

For more information, see *Image Analysis [369]* to Image Analysis.

13.3.4.2.2.2 Frame

In this step you can define one or more measurement frames. Only the area within the measurement frames will be analyzed. Furthermore you can define how the analysis treats objects that are cut by the border of the image or the frame.

Parameter	Description
Interactive	**Activated:** The measurement frame definition can be changed interactively while the measurement program is running in **Analyze Interactively** Mode.
Tool bar	
- Select Frame	Use this to select measurement frames that you have already created. To select a measurement frame, click inside it. To select several measurement frames, hold down the *Ctrl* key and click inside the desired measurement frames. Once you have selected a measurement frame, you can change its size. Delete frames with the *Del* key.
- Draw Rectangle	Enables you to create a rectangle as a measurement frame in the current image.
- Draw Circle	Enables you to create a circle as a measurement frame in the current image.
- Draw Contour (Polygon)	Enables you to create a contour as a measurement frame in the current image.
- Remove All Frames	Removes all drawn-in measurement frames in the current image.
Maximize circle	Only active if you have defined precisely one circle. **Activated:** Maximizes the drawn-in circle to the full image size. In the case of rectangular images the circle is adjusted to the shorter side.
Center circle	Only active if you have defined precisely one circle. **Activated:** Centers the drawn-in circle to the full images size.
Mode	Here you can select how you want the measurement frame to be applied. The following modes are available:
- Inside Only	Measures only those objects, that are lying completely within the measurement frame. Objects that are touching the frame or are intersected by the frame are not analyzed.
- Cut at Frame	Measures all objects that are lying within the measurement frame. Objects that are intersected by the measurement frame are measured precisely up to the measurement frame.
The following fields are only active if you have selected a drawn-in graphic element:	
Left	Here you can enter the start point for the frame on the X axis in pixels.
Top	Here you can enter the start point for the frame on the Y axis in pixels.
Width	Here you can enter the width of the measurement frame in pixels.
Height	Here you can enter the height of the measurement frame in pixels.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard.

For more information, see *Image Analysis [▸ 369]* to Image Analysis.

13.3.4.2.2.3 Automatic Segmentation

In this step you can choose the segmentation method to be applied and set parameters for the segmentation of the objects that you want to measure.

Parameter	Description
Execute	**Activated:** Sets the defined threshold values when the measurement program is run.
Interactive	**Activated:** The defined threshold values can be changed while the measurement program is running in **Analyze Interactively** mode.
Classes	Here you can select the class for which you want to define the segmentation.
Ring Element Class	This additional parameter is only available if you have selected the **Zone of Influence** (ZOI) method.
- Ring Distance	Distance from surface of the primary object. Negative values mean that the ring starts at the defined distance within the primary object.
- Ring Thickness	Defines the thickness of the ring.
ZOI Class	This additional parameter is only available if you have selected the **Zone of Influence** (ZOI) method.
- ZOI Width	Allows you to set the distance of border of the ZOI from the border of the ring, or the main object, respectively.
Apply	Executes the selected segmentation method after a parameter change. Time consuming segmentation methods like Intellesis Trainable Class Segmenter are not run again automatically when a parameter is changed.
Select	Opens the selection of segmenters. For more information, see *Segmentation Method Selection [▸ 784]*.

The following parameter are visible depending on the selected segmentation method.

Model Section

Parameter	Description
Select Model	Opens the dialog to choose a segmentation model, see also *Select Model dialog [▸ 785]*.
Reset	Resets/ Deletes the selected model.

Smoothing section

Parameter	Description
Smoothing	Here you can select how you want to smooth the image before the threshold values are set. The following methods are available:
- None	The image is not smoothed.
- Low Pass	Applies the Low Pass Method.
- Gauss	Applies the Gauss Method.
- Median	Applies the Median Method.
Size	Only visible, if you have selected **Low Pass** or **Median**. Enter the size of the filter matrix in the X and Y direction using the slider or input field.
Sigma	Only visible, if you have selected **Gauss**. Enter the sigma value using the slider or input field.

Variance section

Parameter	Description
Variance	Set the kernel size which is used to calculate the variance value of one pixel with its neighboring pixels using the slider or input field.

Thresholds section

Parameter	Description
Thresholds	Define the lower and upper threshold for the variance using the slider or input field.

Invert

Parameter	Description
Invert	**Activated**: Uses the inverted areas of the histogram as were previously defined.

Dilate

Parameter	Description
Dilate	Dilate the segmented area using the slider or input field. (Value given in pixels).

Subtract BG section

Parameter	Description
Subtract BG	Here you can select what kind of background subtraction is performed. Only visible if **Segmentation with Background Subtraction** is selected.

Parameter	Description
- None	No background subtraction is performed.
- Rolling ball	The rolling ball background subtraction is performed.

Sharpen section

Parameter	Description
Sharpen	Here you can select how you want to improve the sharpness of the image before the threshold values are set. The following methods are available:
- None	The sharpness of the image is not changed.
- Delineate	Applies the Delineate Method.
- Unsharp Masking	Applies the Unsharp Masking Method.
Threshold	Only visible, if you have selected **Delineate**. Enter the threshold value for edge detection using the slider or input field. The threshold value should correspond roughly to the gray value difference between objects and the background.
Size	Only visible, if you have selected **Delineate**. Enter the size of the edge detection filter using the slider or spin box/input field. The value should correspond to the size of the transition area between objects and the background.
Strength	Only visible, if you have selected **Unsharp Masking**. Enter the strength of the Unsharp Masking using the slider or input field. The higher the value selected, the greater the extent to which small structures are enhanced.

Threshold section

Here you can define the threshold values for the selected class in the **Classes** list.

Parameter	Description
Reset	Resets all threshold value settings.
Undo	Undoes the last change made to the threshold values.
Redo	Restores the last undone change to the threshold values.
Color Model	Only visible if the image is a color image. *Color Model [▸ 786]*
- RGB	In RGB Mode you can define the threshold values for the red, green and blue color channels.
- HLS	In HLS Mode you can define the threshold values for hue, saturation and lightness.
Histogram	In the histogram you can change the lower and upper threshold value for the activated value. Drag the lower or upper adjustment handle or shift the entire highlighted area between the lower and upper threshold value.

Parameter	Description
Threshold Definition	
- Click	Click in the image on the regions that you want to define as objects.
- Automatic	The threshold values are determined automatically from the histogram. During setup only the part of the image displayed in the viewport is taken for the calculation of the threshold (only for method RGB).
Pick Behavior	Only visible, if you have selected **Click** for **Threshold Definition**.
- **+**	Enables you to expand the currently segmented regions by the gray values/colors of the objects subsequently clicked on.
- **-**	Enables you to reduce the currently segmented regions by the gray values/colors of the objects subsequently clicked on.
Tolerance	Only visible, if you have selected **Click** for **Threshold Definition**. Enter the tolerance range by which the gray/color value read out when you click is expanded to define the threshold value, using the slider or input field.
Neighborhood	Only visible, if you have selected **Click** for **Threshold Definition**. Enter a neighborhood range around the pixel clicked on, using the slider or input field. The threshold value is calculated from the average of the gray/color values in this neighborhood range.
Method	Only visible, if you have selected **Automatic** for **Threshold Definition**. Select the method from the dropdown list that you want to use for the automatic calculation of the threshold values. The following methods are available:
- Otsu	For all possible threshold values, the Otsu method calculates the variance of intensities on each side of the respective threshold. It minimizes the sum of the variances for the background and the foreground.
- Maximum Peak	Separates background and foreground pixels at the maximum value of the histogram.
- Iso Data	The threshold value lies in the middle between two maximums in the histogram.
- Triangle Threshold	The threshold value is calculated from the sum of the average and three times the sigma value of the histogram distribution.
- Three Sigma Threshold	

Info

After the automatic calculation of the threshold values you can further modify the threshold values found interactively by selecting **Click** for threshold value definition.

Min. Confidence Section

Parameter	Description
Min. Confidence (%)	Sets the minimum value for the confidence that a certain pixel belongs to the segmented class. The default value is 51.

Minimum Area section

Parameter	Description
Minimum Area	Enter the minimum area in pixels that an object must have in order to be segmented, using the slider or input field.

Hole section

Parameter	Description
Min. Hole Area	Enter the minimum area in pixels of the holes of the detected region using the slider or input field. This input is synchronized with the input for **Minimum Area** which must not be smaller than **Min. Hole Area**.
Fill all Holes	**Activated:** Fills holes in segmented objects.

Binary section

Parameter	Description
Binary	Performs morphological operations on the segmented (binary) image.
- None	No operation is performed.
- Open	Opening performs first erosion and then dilation. The effect is smoothing and removing of isolated pixels.
- Close	Performs first dilation and then erosion. The effect is smoothing of the objects and filling of small holes.
- Dilate	Enlarges the boundaries of segmented regions. Areas grow in size and holes within the regions become smaller.
- Erode	Erodes boundaries of the segmented regions. The areas shrink in size and holes within the areas become larger.
Count	Enter how often the selected binary operation is performed, using the slider or input the field.

Separate section

Parameter	Description
Separate	Here you can select whether you want to process the image further after segmentation. Objects that are touching one another can be separated using different methods.
- None	Objects are not separated.

Parameter	Description
- Morphology	This method separates objects by first reducing and then enlarging them, making sure that once objects have been separated they do not merge together again.
- Watersheds	Using this method you can separate objects that are roughly the same shape. This method may however result in the splitting of elongated objects.
Count	Enter how often the method is applied successively to the result at the location of the separation, using the slider or input field.

Suppress Invalid section

Parameter	Description
Suppress Invalid	**Activated:** Discards invalid pixels at the border of the image.

Suppress Border

Parameter	Description
Suppress Border	**Activated:** Suppresses the border pixels which might be incorrect, as areas outside of the image are filled with zeros. Excluded area depends on the kernel size used.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard.

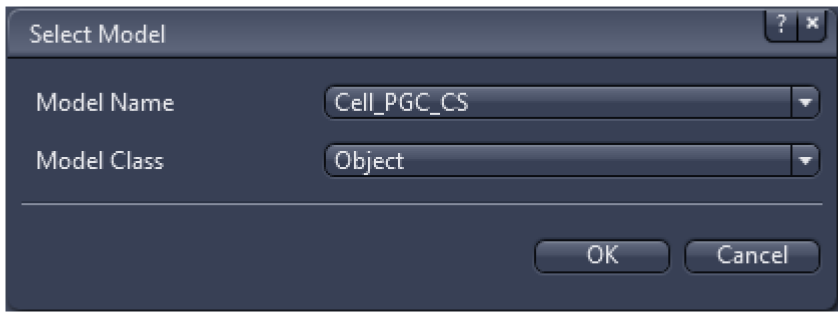
For more information, see *Image Analysis [369]* to Image Analysis.

13.3.4.2.2.3.1 Segmentation Method Selection

Parameter	Description
Segmentation Source	Here you can select the segmentation source. Depending on the selected segmentation source, the functionalities in the **Image Analysis Wizard** change accordingly, see *Automatic Segmentation [779]*. **Note:** This does not work for intensity features as region filter.
- From Image Channel	Uses the channel of the multi-channel image defined in step Classes for segmentation of the selected region class.
- From Measurement Frame (s)	Takes the regions from the measurement frame. You can modify the region in the interactive segmentation step.
- Remaining to Frame	Takes the regions from the measurement frame when the regions of the other classes are subtracted from the measurement frame.

Parameter	Description
- Take from parent regions	Takes the regions that fulfill a certain condition, defined in step Region Filter, from the parent regions.
Image Segmenter	Here you can choose which segmentation method will be applied.
- Segment by global thresholding	A global threshold is applied to the image channel for image segmentation.
- Segmentation with Background subtraction	A background subtraction is performed prior to a threshold-based image segmentation.
- Segment binary images	Segments binary images.
- Variance-based thresholding	Image segmentation via variance-based thresholding. Detects objects where there is a strong change in pixel intensities independent of the absolute intensity.
- Dynamic thresholding	Dynamic thresholding or adaptive thresholding calculates a local threshold for small regions of the image. This is especially helpful for inhomogeneous illumination or background structures.
- Intellesis Trainable Class Segmenter	Uses machine-learning algorithms to segment the selected class by applying a trained Intellesis model.

13.3.4.2.2.3.2 Select Model dialog



Parameter	Description
Model Name	Here you can select a model. Only models trained on one channel images are shown here because only those can be used to segment a specific class assigned to a specific channel.
Model Class	Here you can select the model class.
OK	Sets the model name and class.
Cancel	Cancels the model selection and closes the dialog.

See also

Automatic Segmentation [779]

13.3.4.2.2.3.3 Color Model

RGB

Here you can set the RGB channel threshold values.

Parameter	Description
Red	Activates the red channel in the Expander **Histogram**.
Green	Activates the green channel in the Expander **Histogram**.
Blue	Activates the blue channel in the Expander **Histogram**.
Low	Here you can enter the lower threshold value for the corresponding channel.
High	Here you can enter the upper threshold value for the corresponding channel.
Invert	Swaps the **Lower** and **Upper** values for the corresponding channel.
Full Range	Sets the **Low** value to 0 and the **High** value to the maximum possible gray value for the corresponding channel.

HLS

Here you can set the hue, lightness and saturation threshold values.

Parameter	Description
H **Hue**	Activates the hue in the Expander **Histogram**.
L **Lightness**	Activates the lightness in the Expander **Histogram**.
S **Saturation**	Activates the saturation in the Expander **Histogram**.
Low	Here you can enter the lower threshold value for hue, lightness and saturation.
High	Here you can enter the upper threshold value for hue, lightness and saturation.
Invert	Swaps the corresponding **Lower** and **Upper** values.
Full Range	Sets the corresponding **Lower** value to 0 and the **Upper** value to the maximum possible value.

13.3.4.2.2.4 Region filter

In this step you can define the conditions under which you want an object to be measured.

Parameter	Description
Execute	**Activated:** Uses the measurement conditions when the measurement program is run.
Interactive	**Activated:** The measurement conditions can be changed while the measurement program is running in **Analyze Interactively** mode.
Classes	Here you can select the class for which you want to define the conditions.
Edit	Opens the *Region Filter Editor [▸ 787]*.
Copy to All	Copies the defined region filters to all classes.
Conditions	If you have defined one or more blocks with conditions in the **Region Condition Editor**, you can select the block for which you want to set the condition. Select the relevant block and set the maximum/minimum values either by clicking on the objects in the image you want to include in the measurement, or by entering the maximum/minimum values separately.
Undo	Undoes the last change made to the condition.
Redo	Restores the last undone change to the condition.
Reset	Resets all settings for the conditions.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard.

For more information, see *Image Analysis [▸ 369]* to Image Analysis.

13.3.4.2.2.4.1 Region Filter Editor

All features in the list of selected features will be calculated during image analysis. The results are displayed in the results table for all detected objects of the same class. The columns of the features are sorted according to the order they appear in the **Selected Features** list.

The results of the settings you set here will be displayed in the **Image Analysis Wizard** in the last step in the table with results on the right side. For more information, see *Results Preview [▸ 795]*.

For a detailed description of the individual features, see *Measurement Features [▸ 397]*.

Parameter	Description
Selected Features for Condition	In this list, the features that you have selected for the condition are displayed block by block. All features in a block are "And"-linked for the condition, i.e. an object is only measured if the values of each individual feature fall within the defined range.

Parameter	Description
Add Block	Adds an "**Or**" block. If several "Or" blocks have been defined, an object is measured if it meets the condition in at least one block.
Clear Block	Deletes all features in an "**Or**" block.
Remove Block	Deletes the selected "**Or**" block.
Remove All	Deletes all "**Or**" blocks.

Parameter	Description
Search Features	Searches for features by name.
Drop down menu	Double-click on the feature or click the **+** symbol to add the feature to the list of selected features on the left.
- All	Lists all available features.
- Geometric Features	Lists only the subset of geometric features.
- Intensity Features	Lists only the subset of intensity features.
- Image Features	Lists only the subset of image features.
- Position Features	Lists only the subset of position features.
- Geometric Features Unscaled	Lists only the subset of unscaled geometric features.

Parameter	Description
+ **Add**	Click on the button to select a feature for the measurement.

13.3.4.2.2.5 Interactive Segmentation

In this step you can post-process the segmented objects interactively. You can modify the results of the automated segmentation when you use **Run Interactive** to analyze your image data.

Parameter	Description
Interactive	**Activated:** The segmented objects can be post-processed interactively while the measurement program is running.
Classes	Here you can select the class whose objects you want to process.

Edit Region section

With the tools below you can modify the objects of the currently selected class.

Parameter	Description
Draw	Draw new objects of the selected class.

Parameter	Description
Erase	Using this button you can erase parts of an object. Holding down the left mouse button, outline the parts of the object that you want to erase. Right-click to erase these parts of the object.
Cut	Use this button to separate connected objects. Holding down the left mouse button, draw in the separation line between the objects. Right-click to cut the objects.
Merge	Use this button to connect objects. Holding down the left mouse button, outline the parts of the object that you want to merge. Right-click to merge the objects.
Fill	Fills a hole. To fill a hole, left-click on the hole.
Remove	Removes a drawn in object by clicking on it.
Remove All	Deletes all drawn in objects.
Tool bar	
- Rectangle	Enables you to add a rectangular object or cut a rectangular region from an object.
- Circle	Enables you to add a circular object or cut a circular region from an object.
- Contour	Enables you to add an object or cut a region from an object.
- Contour (Spline)	Enables you to add an object or cut a region from an object.
- Active Contour	Enables you to add an object or cut a region from an object.
- Polyline Region	Enables you to add a line-object.
- Point	Enables you to add a point object.

Region Growing section

Only visible for images with a size smaller than 10000 x 10000 pixel.

Parameter	Description
Mode	
-	Click on areas in the image you want to add to the selected object class.
-	Click on areas in the image you want to remove from the selected object class.

Parameter	Description
Intensity	Here you can enter a tolerance value for the intensity using the slider or input field. The tolerance value specifies how much the intensity of a pixel may deviate from the average intensity of the object in order to still "grow" to become part of the object.
Color	Only active if your input image is a color image. Here you can enter a tolerance value for the color using the slider or input field. The tolerance value specifies how much the color value of a pixel may deviate from the average color value of the object in order to still "grow" to become part of the object.
Fill	Fills holes that are created during region growing.

Post Processing section

Only visible for images with a size smaller than 10000 x 10000 pixel.

Parameter	Description
Region Filter	Reapplies the region filter you defined in the previous step to the post-processed image.

Parameter	Description
Undo	Undoes the last action.
Redo	Restores the last undone action.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard.

For more information, see *Image Analysis [▸ 369]*.

13.3.4.2.2.6 Features

In this step you select the measurement features.

Features you have defined are available from within the **Feedback Experiment**. Any time you change the image analysis settings, you need to reload the ***.czias** file in the feedback script editor to activate the changes. For more information, see *Workflow Experiment Feedback [▸ 240]*.

Parameter	Description
Interactive	**Activated**: The features can be changed interactively while the measurement program is running in **Analyze Interactively** mode.

Parameter	Description
Classes	Here, you can select the class for which you want to define measurement features. For each class there are two entries for which you can define features. The first entry concerns all the objects belonging to the class. The second entry represents an individual object. The results is a statistical value of the measurement feature over all single objects of that class. For example, **Mean Intensity** channel 1 will give you the mean intensity of all single objects. **Area** will give you the sum of all areas of the individual objects.
Region Features	
- Edit	Opens the **Feature Selection** view.
- Copy to All	Copies the defined features to all other class or classes, respectively.
Display	If you activate **Display** in the **Feature Selection** window for a feature, the result of the measurement is displayed next to the corresponding object in the analyzed image.
Annotations	
- Edit	Opens the **Feature Selection** window.
- Copy to All	Copies the annotations to all other classes.
Annotations	Only visible if a "child" class is active. The list shows all annotations that are drawn in for the current class.
Annotation Options	
- Color	Allows you to select the color for the region annotations.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard.

For more information, see *Image Analysis [▸ 369]* to Image Analysis.

13.3.4.2.2.6.1 Features of individual regions

For a description of individual measurement features, see *Measurement Features [▸ 397]*.

Parameter	Description
Search Features	Here you can enter parts of the name of the feature that you are looking for. The features in which the entered character string occurs are listed. Select a type of feature according to which you want the features to be filtered from the dropdown list.
- All	All features are listed.

Parameter	Description
- Geometric Features	All geometric features are listed.
- Intensity Features	All features that analyze intensity values are listed.
- Image Features	All features that contain meta information about the measured image are listed.
- Position Features	All features that describe the position are listed.
- Geometric Features Unscaled	All features that describe unscaled geometric features.
- Position Features Unscaled	All features that describe unscaled positions are listed.
- Polygon-based Features	All features polygon-based features are listed.
- Statistical Features	All features that can be used for plotting in a heatmap (i.e. that provide statistical values suitable for heatmap plotting) are listed.

Parameter	Description
Selected Features	Displays all available features that can be calculated for each object during image analysis.
- Name	Name of the selected feature(s)
- Display	**Activated**: The value of the feature for each object is displayed in the analyzed image.
- Copy	Only visible for **Classes** (collection of objects) and if more than one class exists. If the **Copy** column is empty, the selected feature is not copied to any result table. Copies the selected feature also in the result table of **CLASSES** in the next higher hierarchy level. Copies the selected feature also in the data table of **CLASS** in the next higher hierarchy level. Copies the selected feature also to the data table of the first **CLASSES** element of the same hierarchy level. Copies the selected feature also to the data table of the first **CLASS** element of the same hierarchy level. Copies the selected feature also in the data table of the first **CLASSES** element on the next higher hierarchy level. Copies the selected feature also in the data table of the first **CLASS** element on the next higher hierarchy level.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard.

13.3.4.2.2.6.2 Annotations

Parameter	Description
Search Features	Here you can enter parts of the name of the feature that you are looking for. The features in which the entered character string occurs are listed. Select a type of feature according to which you want the features to be filtered from the dropdown list.
- All	All features are listed.
- Geometric Features	All geometric features are listed.

Parameter	Description
Add	Click on the button to select a feature for the measurement.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard.

13.3.4.2.2.6.3 Features of all regions

For a description of individual measurement features, see *Measurement Features [▸ 397]*.

Parameter	Description
Search Features	Here you can enter parts of the name of the feature that you are looking for. The features in which the entered character string occurs are listed. Select a type of feature according to which you want the features to be filtered from the dropdown list.
- All	All features are listed.
- Geometric Features	All geometric features are listed.
- Intensity Features	All features that analyze intensity values are listed.

Parameter	Description
- Image Features	All features that contain meta information about the measured image are listed.
- Position Features	All features that describe the position are listed.
- Geometric Features Unscaled	All features that describe unscaled geometric features.
- Statistical Features	All features that can be used for plotting in a heatmap (i.e. that provide statistical values suitable for heatmap plotting) are listed.

Parameter	Description
Selected Features	Displays all available features that can be calculated for each object during image analysis.
- Name	Name of the selected feature(s)
- Display	**Activated**: The value of the feature for each object is displayed in the analyzed image.
- Copy	Only visible for **Classes** (collection of objects) and if more than one class exists. If the **Copy** column is empty, the selected feature is not copied to any result table. Copies the selected feature also in the result table of **CLASSES** in the next higher hierarchy level. Copies the selected feature also in the data table of **CLASS** in the next higher hierarchy level. Copies the selected feature also to the data table of the first **CLASSES** element of the same hierarchy level. Copies the selected feature also to the data table of the first **CLASS** element of the same hierarchy level. Copies the selected feature also in the data table of the first **CLASSES** element on the next higher hierarchy level. Copies the selected feature also in the data table of the first **CLASS** element on the next higher hierarchy level.

Parameter	Description

Parameter	Description
Add	Click on the button to select a feature for the measurement.

Parameter	Description
Next	Moves on to the next step of the wizard.
Back	Moves back to the previous step of the wizard.
Cancel	Cancels the wizard.

13.3.4.2.2.7 Results Preview

In this step you will see the preview of the measurement result. The results table contains only the measurements performed in the current view port. The measured image is displayed in the *Analysis View [▸ 855]*.

Parameter	Description
Classes	Here you can select the class for which you want to see the measured features. For each class there are two entries: The "parent" class, which shows the features for all objects together, and the "child" class, which shows the features for each individual object.
Highlight Box	
- Color	Allows you to set the color of the highlight box surrounding the selected object in the image.
- Line Width	Allows you to set the line width of the highlight box around the selected object in the image.

Parameter	Description
Enable chart	**Activated:** Displays a chart with measurement results in the **Analysis View** by default. **Deactivated:** Displays no chart in the **Analysis View** by default.
Chart Type	Sets the default chart type that will be displayed in the **Analysis View**. With the two buttons, you can either select the **Scatter Chart** or the **Histogram Chart** as default.
X-Axis	Selects the default feature that is displayed on the x-axis of the chart in the **Analysis View**. The elements displayed in the drop down menu depend on the previously defined measurement features.
Y-Axis	Only available for the scatter chart. Selects the default feature that is displayed on the y-axis of the chart in the **Analysis View**. The elements displayed in the drop down menu depend on the previously defined measurement features.
Multiple Scenes	Only available for multi-scene images. **Activated**: Sets as the default chart a histogram/ scatter chart that contains the data points of all scenes.
Time Series	Only available for time series images. **Activated**: Sets as the default a chart that displays the analysis results of the selected class in a time series chart.
Heatmap	Only available for experiments with multi well/ multi chamber plates (multiple scenes). **Activated**: Sets as the default chart a heatmap of the well plate.
Back	Moves back to the previous step of the wizard.
Finish	Saves the created analysis setting and ends the wizard.
Cancel	Cancels the analysis setting and closes the wizard.

Table with results on the right side

The results in this table depend on the settings you made in the **Feature Selection** dialog. The table contains all selected features for the highlighted class/classes.

Parameter	Description
ID of the parent	Allows to identify for this object, which subset of objects belong to the same "parent" object.
ID	Default feature of each object. It serves as an identifier for each individual object, but also for the group of objects in the statistics-level (Classes).
Area [µm^2]	If a "child"-class is selected, **Area** gives the Area of each individual object. If a "parent"-class is selected, **Area** gives the sum of the areas of all individual objects in the "child"-class.

For more information, see *Image Analysis [▸ 369]*.

13.3.5 Tools on Applications Tab

13.3.5.1 arivis Vision4D Tool

This tool enables you to send an image to the arivis Vision4D software and open it there with an analysis pipeline selected in **ZEN**. The input image for the tool is always the currently opened image.

Parameter	Description
Options	
– Show Sample Pipelines	**Activated:** Displays sample pipelines in the directory below.
– Add Pipeline Directory	Opens a browser to select and add a folder with pipelines you created in Vision 4D to the list of directories below.
– Remove Pipeline Directory	Removes the selected pipeline directory in the list below. **Note:** You can only remove whole directories / top level folders which you have imported. Removing subfolders or individual pipelines from the list is not possible!
– Update Pipeline List	Updates the pipeline directories.
Recently used	Displays a list of the most recently used pipelines.
Search	Searches for a pipeline using the input field.
Cancel Search	Cancels the current pipeline search.
Open and Apply	Opens Vision4D with the current image and the selected pipeline.

13.3.6 Tools in Right Tool Area

13.3.6.1 Images and Documents Tool

Parameter	Description
List of opened images and documents	Here you find a list of all images and documents which are currently opened in the **Center Screen Area**. The disk symbol with a small warning sign means that you changed and/or have not saved the chosen image or document.
Save	Saves the chosen file. **Save as** dialog will open if you have not saved the file yet.

Parameter	Description
Quick Export	Automatically exports the active image with the default settings of **Single File Export** method to**…/user/pictures** (Windows default folder for images). Images of time series or z-stacks will be automatically exported with the default settings of **Movie Export** method to**…/user/movies** (Windows default folder for movies).
Close	Closes the active image or document.
Text View	Shows only the text of the file .
Thumbnail View	Shows a small preview image (thumbnail) of the file.
Big Preview	Shows a preview image of the file.

13.3.6.2 Microscope Tool

Parameter	Description
Objective List	Here you can easily switch between the objectives and pre-magnification. The color bar on the objective buttons indicates the color for the respective stage limit indicator inside the **Navigation** tab. If you select **autocorr** objectives (motorized correction collar) you can additionally adjust the relevant settings like **Correction Mode, Bottom Thickness** or **Imaging Depth**.

13.3.6.3 Stage Tool

⚠ **CAUTION**

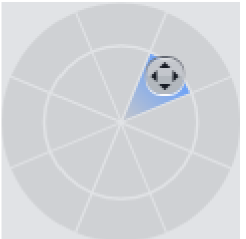
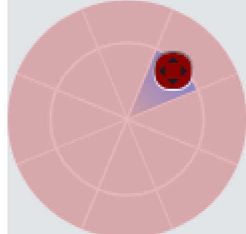
Risk of Crushing Fingers

The drive of a microscope stage with a motorized horizontal stage axis (stage drive) is strong enough to crush fingers or objects between the stage and nearby objects (e.g. a wall).

- Remove your fingers or any objects from the danger area before moving the stage drive.
- Release the joystick immediately to stop the movement.

This tool enables you to navigate the sample in a microscope equipped with a motorized stage. You can use the **Navigation Circle** (software joystick) to move the stage or enter the coordinates directly.

Parameter	Description
Navigation Circle	Enables you to move the stage freely in the X and Y direction and in both diagonal directions. To move the stage, drag the **Navigation Circle** icon in the desired direction. If released, the icon snaps back to the **Navigation Circle** center and the stage stops.

<table>
<tr><th>Parameter</th><th>Description</th></tr>
<tr><td></td><td>The Navigation Circle allows four speeds:
▪ Normal modes:
– Inner segments: Slow
– Outer segments: Medium

▪ High-speed modes:
– Inner segments: Fast
– Outer segments: Very Fast

To enter the high-speed mode, right-click the Navigation Circle icon. The Navigation Circle turns red. To return to normal speed, right-click the Navigation Circle icon again.</td></tr>
<tr><td>Stop</td><td>Stops any stage movement immediately.
Use this button if you entered X-Position and/or Y-Position and wish to interrupt the stage movement immediately (e.g. to prevent a collision).</td></tr>
<tr><td>X-Position, Y-Position</td><td>Specifies the target coordinates for the stage movement.
The stage starts moving immediately after the coordinates have been entered and confirmed; either by pressing the Return key or by clicking anywhere outside the current input field.</td></tr>
</table>

Info

You can also control the **Navigation Circle** and thus the motorized stage with the keyboard. To activate keyboard control left-click anywhere inside the segmented **Navigation Circle**. To change between the two speed modes, right-click the central**Navigation Circle** icon.

- To move the stage at the lower speed, use the arrow keys (diagonal movements are also possible).
- To move the stage at the higher speed, use **Shift + Arrow** keys.

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Speed	Sets the moving speed of the stage in percent (100%=maximum possible speed). Note that the speed setting does not change the speed graduation of the SW joystick.
Acceleration	Sets the acceleration of the stage in percent (100% = maximum acceleration value).
X/Y-Position	
- Set Zero	Sets the current position as the new zero point for the x/y coordinates.
- Calibrate	**CAUTION! Risk of Crushing Fingers.** Performs an automatic stage calibration. For this the stage moves to the limit switches to determine the zero points in the x and y direction and then returns to its starting position, which is now defined with its absolute coordinates.

Marks section

This section shows a list where you can define **X** / **Y** positions (optional z value), so called marks. It is also possible to import a list of positions from the list into an experiment including the '**Tiles**' tool.

13.3.6.4 Focus Tool

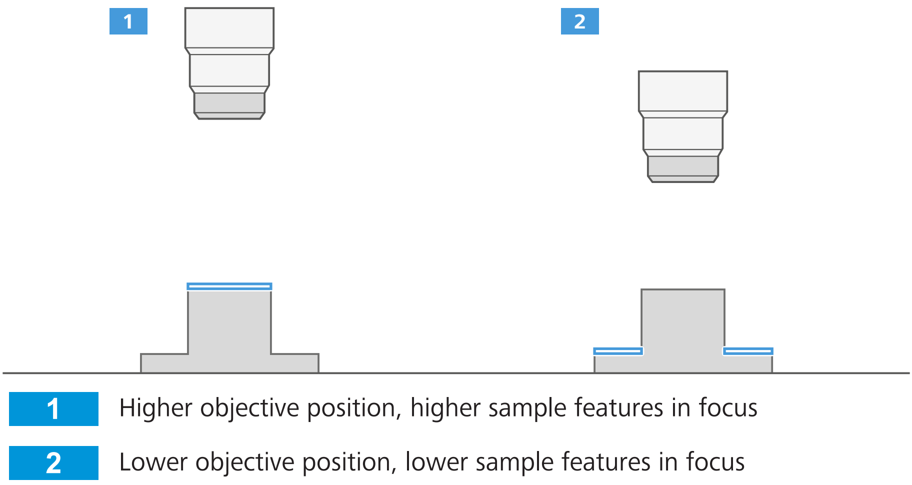
⚠ CAUTION

Risk of Crushing Fingers

The drive of a microscope stage with a motorized vertical axis (focus drive) is strong enough to crush fingers or objects between the stage and the microscope stand.

- Remove your fingers or any objects from the danger area before moving the focus drive.
- Release the joystick immediately to stop the movement.

This tool changes the vertical distance (i.e. Z direction) between stage and objective. This enables you to focus the sample, or, for a sample with an uneven surface, to focus the area of interest.



1	Higher objective position, higher sample features in focus
2	Lower objective position, lower sample features in focus

Parameter	Description
Current	Displays the stage position in µm Initially, when you use the **Focus** tool for the first time after switching on the microscope, the exact position of the stage is not known. Therefore, the position indicated by **Current** is initially set to zero. If you enter a value, the stage moves by the entered amount relative to the current position. If you want to move the focus to an absolute position, you must first click **Home** to move the focus to one of the end positions. The value of **Current** is set to this known position. You can then enter an absolute position.
Position control	Enables you to set the stage position. You can either use the **Navigation Bar** to move the stage up or down or you can enter the target position in the **Current** input field.
– Navigation Bar	Enables you to move the stage freely in Z direction To move the stage, drag the **Navigation Bar** button in the desired direction. If released, the **Navigation Bar** button snaps back to the center and the stage stops. The **Navigation Bar** allows four speeds. Normal modes: ▪ Inner segments: Slow ▪ Outer segments: Medium High-speed modes: ▪ Inner segments: Fast ▪ Outer segments: Very Fast To enter the high-speed mode, right-click the **Navigation Bar** button. The **Navigation Bar** turns red. To return to normal speed, right-click the **Navigation Bar** again.
– Current	Defines the target position of the stage in µm. The stage starts moving immediately after the coordinates have been entered and confirmed by pressing the *Enter* key or by clicking anywhere outside the **Current** input field.
– Stop	Stops any stage movement immediately.
Backlash Correction	Activated: Enhances the positional accuracy by performing an extra movement. When activated the focusing takes slightly longer
Handwheel on	▪ Activated: Turning the handwheel also adjusts the focus ▪ Deactivated: The handwheel is deactivated: turning it does not affect the focus
Step Size	Defines the difference in µm by which the stage moves at each step. Indirectly this defines the speed of the stage movement. The **Step Size** also determines the accuracy of the focus position.

Parameter	Description
Home	Moves the focus to one of the end positions. The value of **Current** is set to this known position. This ensures that the position shown as **Current** corresponds to the actual stage position.
Work	Moves the stage back to the position it was in before using the **Load** button (i.e. the work position) If you have moved the stage (e.g. using the **Navigation Bar**) after moving it into the load position, the work position is lost and the **Work** button will not work.
Load	Increases the distance between objective and stage by 8,000 µm This aids you in exchanging the sample. After exchanging the sample, you can move the stage back into its work position by using the **Work** button. Make sure not to move the stage (e.g. using the **Navigation Bar**) after moving it into the load position. Otherwise, the previous position is lost and the **Work** button will not work.
Measure	This function allows to measure distance in Z direction.
Z-Position	Specifies which position of the motorized z drive is used as the origin (zero value)
– Set Zero	Sets the current focus position as the origin (zero value)
– Calibrate	Performs an automatic calibration

13.3.6.5 Definite Focus Tool

Parameter	Description
Status	Displays the actual status of the device, e.g. Standby or Monitoring.
Find Surface	Tries to find the surface of the cover glass and adjusts the focus position accordingly. If the signal is not strong enough, the actual focus position remains.
Store Focus	Sets (and saves) the current focus position as the stabilizing position. Note that if you change an objective, the saved position will be deleted.
Recall Focus	Restores the saved focus position.
Lock Focus	If activated, Definite Focus holds the current focus distance by starting a continuous focus stabilization.

13.3.6.6 Incubation Tool

In the Incubation Tool you can define and control parameters for temperature, atmosphere and the Y-Module. The available parameters depend on which components you have configured on your system.

Info

The symbols behind measured values indicate if the measured and the set values are

- the same = green check mark,
- different = red or blue triangle with exclamation point, or
- not activated = blue circle with question mark.

Parameter	Description
Temperature	Here you can control up to 4 independent heating channels that are linked to certain devices (e.g. incubator XL, heating insert P, objective heater etc.). The devices are assigned to different channels in the Micro Tool Box (MTB).
- Heating/ Cooling	**Activated:** The channel will be used for the experiment. Under **Setpoint** you can set the temperature of the channel in °C. Under **Measured** you see the currently measured value.
- Channel (1-4)	**Activated:** The channel will be used for the experiment. Under **Setpoint** you can set the temperature of the channel in °C. Under **Measured** you see the currently measured value.
- Sensor	Shows the current temperature inside the incubation chamber.
Atmosphere	Here you can define the O2 and CO2 concentration, as well as the temperature for an Air Heater module. Note that the meaning of the symbols behind the measurement values is the same like described above.
- O2 Channel	**Activated:** The O2 channel will be used for the experiment. Under **Setpoint** you can set the O2 concentration of the chamber in percent (1-100%). Under **Measured** you see the currently measured value.
- CO2 Channel	**Activated:** The CO2 channel will be used for the experiment. Under **Setpoint** you can set the CO2 concentration of the chamber in percent (1-100%). Under **Measured** you see the currently measured value. ▪ **Fan Speed**: Sets the rotation speed of the fan.

Parameter	Description
- Air Heater	**Activated:** The air heater will be used for the experiment. Under **Setpoint** you can set the temperature of the air heater in °C. Under **Measured** you see the currently measured value. ▪ **Fan Speed**: Sets the rotation speed of the fan.
- Pump Level	Sets the pump level. **Note:** If **O2 Channel** or **CO2 Channel** is activated and the **Pump Level** is set to 0, a warning is displayed to remind you to set the pump level >0 so that the gas is actually pumped to the probe.
Y-Module	The Y-Module panel allows setting the temperature for two independent modules (thermostats).
- Selected	Here you can select which module you want to control (**Module 1** or **Module 2**).
- Circulator 1-2	**Activated:** The channel will be used for the experiment. Under **Setpoint** you can set the temperature of the channel in °C. Under **Measured** you see the currently measured value. For each module two circulator channels can be activated.

13.3.6.7 Macro Tool

Parameter	Description
Record	Records a new macro. The **Record** button will change to a **Stop** button. Press this **Stop** button to stop the macro recording.
Run	Executes the selected macro.
Stop	Stops the running macro at the active command.
Selection	Displays a list of all existing macros.
– Add	Creates a new macro.
– Delete	Deletes the selected macro.
– Options	With the options dropdown you can create, duplicate, rename, and safe new macro files, or delete existing macros.
Preview	Here you see a preview to the macro program code of the selected macro. Editing the macro here is not possible.
Properties	
– Name	Displays the name of the macro.
– Keywords	Displays keywords for the selected macro. Keywords can also be entered in this text field.

Parameter	Description
– Description	Displays the description for the selected macro. A description can also be entered in this text field.
– Toolbar Configuration	When you click on the button you will enter the **Customize Toolbar** dialog. There you can add macro buttons or functions to the toolbar for a quick access. How you can configure the toolbar is described under *Customizing Toolbar [▸ 237]*.
Macro Editor…	Opens the *Macro Editor Dialog [▸ 622]*.

See also

Right Tool Area [▸ 22]

13.3.6.8 Lasers Tool

The is equipped with a variety of diode and solid state lasers. The lasers can be controlled (switched from Standby mode to On) vie ZEN. The tool displays only lasers which are available with the system. (For LSM 980 only.)

Fig. 70: Lasers tool

1 **Laser Lines [nm]** column

Lists the available laser wavelengths. For Multiphoton Lasers the tunable range and fixed line (if available) of the laser is displayed.

2 **Power** column

Contains the **On/Off** power control buttons one for each laser line. For Multiphoton lasers the ON/OFF effects both, the tunable and the fixed line.

3 **All Lasers Off** button

With one click all still active lasers can be switched off.

4 **Laser Properties** panel

Shows the laser properties of the selected (highlighted) laser line. The listed properties always contain the current wavelength, the status of the laser and the power.

- The status of diode or solid state lasers changes between **Connected**, **Warming up** and **Ready**.
- The status of Multiphoton lasers changes between **Connected**, **Warming up** and **Mode locked**. The laser is ready for use only when in **Mode locked** status.
- The status of Multiphoton lasers also changes when the laser is tuned (**Tuning**), the GDD unit is adapted (**GDD Positioning**) or when the laser is in CW mode (**CW lasing**). In any such status the laser cannot be used for imaging.
- For Multiphoton lasers the panel contains the control for the GDD (group dispersion delay) unit (if available).
- In case two Multiphoton lines are available for imaging the second (typically non tunable) line with its status is displayed at the bottom of the panel.

Prerequisite
✓ The system components are switched on
✓ ZEN is running

1. In order to use a laser for imaging, click **On** in the drop down list in the **Power** column of the desired laser.
   → A warning triangle next to the activated laser line indicates the laser is now on and emits laser light.

Some lasers need up to 1 minute to warm up to their full power level. In this status the laser line is marked in red in the **Channels** Tool and in the **Timed/Interactive Bleaching** Tool. Multiphoton lasers take several minutes to warm up to full power.

All lasers which are needed for an experiment (load experiment), a track configured using **Smart Setup** or when an image is re-used to reproduce the imaging acquisition parameters, are switched on automatically. A laser is also switched on when it is selected in the **Channels** Tool, the **Imaging Setup** Tool, the **Timed Bleaching** Tool or the **Interactive Bleaching** Tool.

Option
Lasers can be set to automatically switch off after 30 minutes of inactivity. This function can be activated/deactivated in **Options | Acquisition | LSM**. Changing this function requires to restart ZEN.

GDD Control
The settings for the GDD unit are predefined when the system is set up. The unit will move to a specific value when the laser wavelength is changed. The wavelength of the multiphoton laser might be changed either in the **Channels** Tool, when loading an experiment or when re-using an image.

The value for GDD can be adapted to the experiments needs. In order to achieve the shortest possible pulses for a given setting (wavelength and objective), move the slider to a different position while scanning in **Live** or **Continuous** mode. When the image intensity is at its maximum, the shortest possible pulses reach the sample for multiphoton excitation. Store the value to use it for the next image acquisition using **Snap** or **Start Experiment**. The value for the current wavelength is displayed next to GDD.

GDD values may vary also with the objective. The pre-set values assume that a large high NA objective is used. The variations between similar types of objectives can be neglected. Hence the settings might only need small adaptions if at all for a given wavelength. The GDD value does not change if a wavelength is chosen for which no value is stored. Restore sets all GDD values back to the factory settings.

13.3.6.9 Cryo Temperature Tool

In this tool you can see the temperature status of the Linkam Cryo stage. When the stage is selected in the MTB configuration and properly connected to the computer, the temperature is logged beginning with the ZEN startup. This logging of data is asynchronous and runs as long as ZEN is active.
Note that if the stage is physically disconnected from the computer, only the last received temperature is logged. Only after a restart of ZEN the current temperature is again logged in a new log file.

Parameter	Description
Temperature Status	Displays the current temperatures.
– Bridge Temperature	Displays the current bridge temperature.
– Chamber Temperature	Displays the current chamber temperature.
– Dewar Temperature	Displays the current dewar temperature.
Temperature Logging	
– Interval	Only visible if **Show All** is activated. Sets the interval for reading the temperature data between 5 to 60 seconds.
– Open record	Opens the record for the logged temperatures. **Current** opens the current log file in ZEN as a .czt file. **Folder** opens the folder where the log file is saved on your computer (C:\Users\user\Documents\Carl Zeiss\ZEN\Documents\TemperatureLogging).
– **Logging**/ **Start Logging**	Shows that the temperature is logged. Click on **Logging** to stop logging the temperature. The button changes to **Start Logging**. Click on **Start Logging** to continue the logging of thee temperature.
Image Temperature Data	
– Export Temperature Data	Opens the temperature data as a table in ZEN.

13.4 Dialogs

13.4.1 Stage/Focus not calibrated Dialog

If you see this dialog, after you have started the software and the hardware was initialized, you should consider to calibrate the stage and focus drive immediately.

The calibration is necessary, if

- a motorized stage and/or focus drive are used, and
- the stage and focus drive are not calibrated.

To start the calibration procedure, simply click on the **Calibrate Now** button.

Risk of Crushing Fingers !

The drive of a microscope stage with a motorized vertical axis (focus drive) is strong enough to crush fingers or objects between the stage and the microscope stand.

- Before starting the calibration procedure, ensure that people stand clear of the instrument and that the full travel range is not obstructed by any objects.

If you skip the calibration, you can calibrate the stage and focus drive afterwards within the **Stage Control** and **Focus Control** dialogs accessible via the **Lightpath** tool, see Stage Control and Focus Control. Make sure that the **Show All** mode is activated, to see the **Calibrate** button within the dialogs.

Note that for fully automated system like Axio Scan the axes are calibrated automatically. The calibration is not necessary in that case.

13.4.2 ApoTome Dialog

Parameter	Description
Recommended Grid	In this section you can set the grid with which you want the ApoTome to be operated.
Automatic Grid Control	Activated by default. **Activated**: The appropriate grid for the selected objective is selected automatically (in the case of ApoTome.2). **Deactivated**: It is possible to select another grid from the dropdown list that is now active, e.g. to create a thicker optical section thickness. A list of recommended objectives and suitable wavelengths can be found here.
Calibration Status	Here you can see whether your ApoTome has been calibrated successfully or whether calibration needs to be performed.
Theoretical Thickness	The theoretical section thickness for the selected filter set and the objective used is displayed here.

13.4.3 ApoTome Settings Dialog

Parameter	Description
Camera	Here you can select the camera you wish to use to acquire your **Apo-Tome** images. As soon as you have selected a camera, **ApoTome** images are generated automatically during acquisition (**Snap**). The selected camera also applies to the **Acquisition** tab.
Live Mode	Here you can choose between the **No Combination**, **Optical Section** and **Conventional Fluorescence** modes for the live image.
Acquisition Mode	Here you can choose between the **No Combination**, **Optical Section** and **Conventional Fluorescence** modes for acquired images.
Phase Images	Here you can choose between no fewer than 3 and no more than 15 phases. Each phase corresponds to a grid position. By default, 5 phases are acquired.
Filter	Here you can set a filter which can be used to filter out residual streaks from the image. You have a choice between no filtering (**Off**) and three strength levels.
Image Normalization	**Activated**: The gray values are extended to the maximum available dynamic range following the calculation, see Normalization .

13.4.4 Add Dye or Contrasting Method Dialog

Here you add dyes and contrast techniques to your experiment. The dyes in the database contain important information that is saved in the image document (e.g. spectral characteristics). This information can be used later during image processing (e.g. deconvolution).

Info

You can add additional dyes to the database with the **Dye Editor** under **Tools > Dye Editor...**.

Parameter	Description
Recently used	Displays the six recently used dyes or contrasting methods in a list. This ensures that you have quick access to the dyes or contrasting methods that you use frequently.
Search	Here you can enter the name or initial letters of the dye or contrasting method that you want to search for. The search results are displayed immediately in the **Dye Database** list or the **Contrasting Methods** list. If no search filter is active, the lists of dyes or contrast techniques are arranged in alphabetical order. If you cannot find a certain dye, try using a related dye name or a general name.

Parameter	Description
Dye Database	Choose fluorescent dyes here. Double click on the dye or click on the **Add** button to add it to the experiment. The left column shows the name of the dye. The right column contains its color and main emission wavelength. The "**Custom**" entry adds a channel to your experiment without any additional information. This means that the resulting image cannot be used for certain processing operations.
Contrasting Methods	Only available for **WF** tracks. Here you can choose a contrasting method. Double click on the contrasting method or click on the **Add** button to add it to the experiment.
Add	Adds the selected dye to the experiment. You can add several dyes/ contrasting methods in a row.
Close	Closes the dialog.

13.5 Image views

The software offers a lot of different image views. The **general image views** are visible for each image. The **specific image views** are available only if the image has the appropriate characteristics (eg multiple channels, Z-stack, etc.) or you have licensed a specific module (e.g. for 3D View). Each image view has general and specific controls which you can use to work with the view.

13.5.1 General image views

These image views are available with any image. Depending on the type of image in question, the **general control elements** may have additional or more limited functions.

13.5.1.1 2D View

This view is the default view for images in the software. For this view the *General View Controls [▸ 887]* are available to you. To open the view's *context menu [▸ 810]*, right-click in the *Center Screen Area [▸ 21]*.

13.5.1.1.1 2D View Context Menu

Menu item	Description	Short cut
Full Screen	Switches to full screen mode. To exit full-screen mode, press *F11* or *ESC*.	*F11*
Zoom Group	Here you have access to the main zoom functions (Dimensions tab > *Zoom section [▸ 888]*).	
Rulers	Shows rulers at the top and left edge of the image.	
Show Floating Scale Bar	Shows a scale bar, which you can position freely in your image.	*Alt + S*
Navigator	Shows the **2D view** Navigator window.	

Menu item	Description	Short cut
Spot Measurement / Focus ROI	This function is only active in the live image or during **Continuous** mode. Shows a region in which the exposure time is measured and the software autofocus is focused.	
Graphics	This function is activated by default. Shows graphic elements that have been drawn into the image, e.g. annotations or scale bars.	
Show Bleach ROI	This function is only visible with FRAP images. Shows graphic elements that were used during acquisition for bleaching (FRAP).	
Grid	Adds a grid to the image.	
Copy Display Settings	Copies the display settings from an image (Display tab).	
Paste Display Settings	Inserts copied display settings into an image (Display tab).	
Paste Display Settings and Channel Colors		
ROI (Region of Interest) | Draw Region of Interest	Draw a certain rectangular region that is of particular interest to you into the image. The ROI is displayed with red boundaries. You can draw several regions into an image.	*Ctrl+ U*
ROI (Region of Interest) | Draw Rotatable Region of Interest	Draw a certain rectangular region that is of particular interest to you into the image. This region can be rotated and is displayed with yellow boundaries. You can draw several regions into an image.	*Ctrl+Shift +R*
ROI (Region of Interest) | Create Subset Images From ROI	Creates new image documents from the selection regions you have drawn in. All dimensions of the image are taken into account here. This function works for both the non rotatable and rotatable ROIs (the red and the yellow regions).	*Ctrl+ Shift + C*
Create Image from View	Creates an image from the current view.	
Paste	Inserts a graphic element into the current image from the clipboard.	*Ctrl + V, Shift + Ins*

13.5.1.2 Gallery View

In this view you see an overview of your multidimensional images. The individual images of the images concerned are presented in a gallery. It is possible to show any combination of dimensions, e.g. channels against time. When you view images for the first time in the **Gallery** view, they are displayed as follows:

Image type	Display
Multichannel image	All the channels present in an image are shown, including the mixed color image.
Time lapse image	All the time points present in an image are shown.
Z-stack image	All Z-planes are shown.
Multichannel & time lapse image	All the time points present in an image are shown. All channels are shown as a mixed color image.
Multichannel & Z-stack image	All Z-planes are shown. All channels are shown as a mixed color image.
Time lapse & Z-stack image	All Z-planes are shown.
Time lapse, Z-stack & multichannel image	All Z-planes are shown. All channels are shown as a mixed color image.

See also

General View Options [▸ 887]

13.5.1.2.1 Gallery Tools Tab

Here you can specify which dimension you want to be displayed on which axis of the Gallery view. To do this, click on the corresponding dimension´s button.

Displayed Dimensions section

Each of the buttons is only visible if the current image contains the corresponding dimension.

Button	Function
Channels	Shows the channels present as individual images.
Z-Stack	Shows the Z-Planes present as individual images.
Time Series	Shows the time points present as individual images.
Chann.& Z (Channels and Z-Stack)	Shows the channels present in relation to the Z-stack images present.
Chann.& Time (Channels and Time Series)	Shows the channels present in relation to the time lapse images present.
Z&Time (Z-Stack and Time Series)	Shows the Z-Stack images present in relation to the time lapse images present.

Options section

Checkbox	Function
Show Dimension Labels	Inserts annotations into each individual image that provide information on the time point or Z-plane.
Invert X/Y axis	This checkbox is only available if the **Show All** mode is deactivated. It is active only if two dimensions are shown in relation to each other (Chann.&Z, Chann.&Time, Z&Time). If activated, this function inverts the X and Y axis of the view.
Show Graphics	Shows graphics / annotations within the images (in case if graphics / annotations are drawn in).
Show Merged	Only visible for multichannel images. Only active if the channels present are shown. Shows the pseudo colored (mixed) images of all channels in addition to the individual images.

Advanced Functions

The following functions are only visible if the **Show All** mode is activated:

X Axis / Y Axis settings

From the first 2 dropdown list you can select which dimension (depending on which dimensions are available in the active image, e.g. channels, z-stack, etc.) will be shown on the X or Y axis (X axis = horizontal direction, Y axis = vertical direction).

In the second dropdown lists you can select wether you want to display all images of each dimension or if you want to display a certain range of images on the X or Y axis. Therefore you find the following options :

Option	Description
All	Displays all images of the active image in the Gallery view.
Subset by Step	If selected, you can enter a step size in the **Step** input field. If 2 steps are entered, only every second image will be shown. In the **Max.** input field you can enter the desired number of images which will be shown. The step size will be calculated automatically.
Subset by Range	If selected, you can adjust a range of images (e.g. from image 4-10) which is displayed in the view. Use the slider or the input fields to enter the desired range.

Image Creation

Here you can directly create images out of the Gallery view. Select the type of image that you want to create from the **Create image from** dropdown list. If you click on the **Create** button the image will be generated and opened in a new image document. The resulting image contains all the information of the input image; the pixel data are not changed. Following options are available:

Option	Function
Gallery View	Creates an image of the current Gallery view. If this option is selected, the option **Gallery Image from** is available. Here you can additionally select a dimension that is not currently displayed (e.g. Single Image will export each single image additionally). The resulting image is always a 24 bit RGB color image. The pixel data of the original image are changed. If the **Burn in graphics** checkbox is activated, all graphics or annotations will be burned into the output images.
Selection Subset	Creates an image from the images that have been selected in the current view. To select an image simply click on the image in the Gallery view. Hold **Crtl**-Key while clicking to select more images at once.
Range Subset	Creates an image from the defined selection range. If this entry is selected, sliders for the selected dimensions appear (**Start**, **End** and **Interval**). Use the sliders to set the selection range you want.

13.5.1.2.2 Gallery Appearance Tab

Only visible if the **Show All** mode is activated.

Parameter	Description
Dimension Labels	Here you can define the font and the style, color, position and size of the text for the dimension details that are shown.
Layout	Here you can set the background color of the **Gallery view** and the distance between the individual images (from 1-10 pixels).

13.5.1.3 2.5D View

In the **2.5D view** intensity values in a two-dimensional image are converted into a height map. Here the highest intensity values are represented by the greatest extension in the Z-direction. Overall this results in a so-called 2.5D or pseudo-3D image.

Info

If you are viewing a multichannel image, you can have the intensity values of the individual channels displayed. To do this, activate or deactivate the desired channels on the **Dimensions** tab.

13.5.1.3.1 2.5D View Tool bars

The tool bars are arranged to the left of and underneath the image area. You can use the tools to control the display of the 2.5D volumes in the image area.

Left Tool Bar

Icon	Parameter	Description
	Top thumb wheel	Enlarges or reduces the image area.
	Rotate	Use this to rotate the 2.5D volume in any way you wish within the space. This is the default mode when you switch to **2.5D view** for the first time.
	Zoom	Use this to increase the zoom factor of the image area.
	Bottom thumb wheel	Rotates the 2.5D volume around the horizontal (X) axis.

Bottom Tool Bar

Icon	Parameter	Description
	Left thumb wheel	Use this to rotate the 2.5D volume around the vertical (Y) axis.
	Bounding Box	Use this to show or hide a bounding box around the 2.5D volume.
	Show X/Y Axis	Use this to show or hide the X/Y axis.
	Show Z Axis	Use this to show or hide the Z axis.
	- **Start View**	Use this to switch back to the start view. A top view of the 2.5D volume is displayed. Lateral movements and the zoom factor are adjusted so that the 2.5D volume can be seen at the center of the image area.
	Right thumb wheel	Use this to compress the 2.5D volume on the (Z) axis perpendicular to the screen plane.

13.5.1.3.2 2.5D Display Tab

On the **2.5D Display** tab you have 4 **Render mode** options for displaying your 2.5D image.

Parameter	Description
Render mode	
- Profiles	Displays the relief divided into a number of profiles with an equal distance. Set the number of profiles using the **Grid distance** slider.
- Grid	Displays the relief overlaid with a grid. This view supports gray levels only. Make the grid more closely or more coarsely meshed using the **Grid distance** slider.
- Filled	Displays the height relief by upwardly discrete, layered pillars.
- Surface	Displays the relief as a continuous, flowing landscape. Make the surface coarser or finer using the **Grid distance** slider.
Invert Z axis	Use this function for images that contain many large, bright regions. **Activated:** Displays the lowest intensity values by means of the greatest extension in the Z direction.
Use palette	**Activated:** Overlays the relief with the pseudo colors that have been set on the **Dimensions** tab.
Show Faces at Side	Only available in the **Surface** render mode. **Activated:** Closes the sides of the relief.
Show plane	**Activated:** Shows two blue, transparent planes in the 2.5D volume. Set the position of the planes using the **X/Y** sliders.
Extract image	To save an individual image in the current view, click on the **Save As** button.

13.5.1.3.3 Series Tab

On the **Series** tab in the **2.5D** view you can create a series of images in the 2.5D view. These series can be played back later as a video clip, for example.

Parameter	Description
Render Series	Here you can select the desired series mode:
- Turn Around X	Here you can define the start/stop angle and the rotation direction around the X axis.
- Turn Around Y	Only visible in the **3D** view. Here you can define the start/stop angle and the rotation direction around the Y axis.
- Turn Around Z	Here you can define the start/stop angle and the rotation direction around the Z axis.

Parameter	Description
- Start/Stop	Here you can define the angle and zoom settings for the start and end position of your series. The intermediate positions are interpolated evenly.
- Position List	Here you can define any number of positions. The positions can each have completely different rotation, zoom and illumination settings.
- Over Time	Only visible in the **2.5D** view. Here you can define the start time point and end time point for a series. All other settings (rotation, zoom, etc.) remain unchanged.
Stored	
Options	Opens the Options shortcut menu.
Apply	Creates a series image with the current settings.
Preview	To obtain a preview of the series, click on **Play**. To end the preview, click on **Stop**.
No. of Frames	Here you can enter or select the number of individual images in the series.

13.5.1.3.4 2.5D Display Options Tab

Only visible if the **Show All** mode is activated.

Parameter	Description
Angle X	Enter the rotation angle in the X direction with a precision of 1 degree using the slider or input field.
Angle Y	Enter the rotation angle in the Y direction with a precision of 1 degree using the slider or input field.
Z Scaling	Enter the Z scaling using the slider or input field.
Ambient	Reduces or increases the intensity of the ambient lighting in the 2.5D view.
Reflection	Reduces or increases the proportion of the ambient light reflected on the relief.
Shine	Reduces or increases the effect of the ambient light shining on the relief.
Light height	Reduces or increases the intensity of the lighting in the 2.5D view. A small distance means a circular light source at the center, while a large distance illuminates the scene evenly.
Reset lights	Resets all settings to the default values.

13.5.1.4 Profile View

In the **Profile** view you can create intensity profiles in your image. The view is divided into 4 quadrants: **Profile window** (top left), **Image window** (top right), **Profile table** (bottom left), and **Interactive Measurements table** (bottom right).

Info

To create an intensity profile of a certain region, select a tool on the **Profile Definition** tab. Use this to draw a line across a structure of interest in the image. An intensity profile of the drawn line is generated automatically and displayed in the **Profile window**. To zoom into an aspect of the Profile window, drag out a rectangular frame using the left mouse button in the Profile window. The selected region is displayed in enlarged form. Right-click to return to the original view.

See also

General View Options [▸ 887]

13.5.1.4.1 Profile Definition Tab

Tool bar

Using the tools you can add one of four different profile tools(**Arrow**, **Polygon**, **Freehand**, or **Rectangle**) to your image. The intensity profile of each tool is shown in the **Profile window**.

Parameter	Description
Select	Changes the mouse pointer to Selection mode. You can use this to select graphic elements in the image.
Clone	Use this to copy the last selected element and insert it at another position in the image.
Arrow	Use this to insert an arrow into the image. The gray levels for each pixel along the line are shown in the **Profile window** in the direction of the arrow.
Polygon	Use this to insert a polygonal measurement line in the original image.
Freehand	Use this to insert a measurement line with a shape of your choice.
Rectangle	Use this to insert a rectangular measurement region. For this tool, the average gray values across the width of the rectangle are shown in the **Profile window** and the **Profile table**.
Keep Tool	**Activated:** Keeps the last selected tool active.
Auto Color	Only visible if the **Show All** mode is activated. **Activated:** Highlights each drawn-in measurement line with a random color.
Stroke Thickness	Here you can enter the line width of the measurement line. For line widths larger than one pixel, the average gray values across the line width are shown in the **Profile window** and **Profile table**.

Parameter	Description
Show profile in graphics	**Activated:**Adds the profile curves to the profile tool drawn into the image. This function is only available for the **Arrow** tool.

Profile Measurements section

Parameter	Description
Normal	Switches the mouse behavior for interacting with the **Profile window** to the standard behavior.
Measurement	Use this to perform a point measurement in the **Profile window**. To copy the measured value into the interactive measurement table, click on the **Add to table** button.
Caliper X	Use this to perform a number of measurements along a certain distance along the X axis of the profile. To adopt the value into the measurement data table, click on the **Add to table** button.
Caliper Y	Use this to perform a number of measurements along a certain distance along the Y axis of the profile. To copy the value into the interactive measurement table, click on the **Add to table** button.
Reset Table	Deletes all values from the interactive measurement data table.
Add to table	Adds the current measurement values of tools used in the Profile window to the interactive measurements table.

Grid Distance section

Parameter	Description
Grid Distance	Adjusts the value with the slider or input field. It defines how the measurement is done: For a grid size of 1, each pixel covered by the profile tool is measured. For a grid size of 2, the average of 2 pixels is measured. For a grid sized of 3, the average of 3 pixels is measured etc. This is useful when measuring line profiles in very large tile images e.g. from Axioscan.

13.5.1.4.2 Profile View Tab

Here you can configure the display for the Profile view.

Show section

Parameter	Description
Profile Table	**Activated:** Shows the profile table.
Int. Measurement	Shows the interactive measurement table.
Values	Currently without function.
Image	Only visible if the **Show All** mode is activated. **Activated:** Shows the **Image window**.

Channel section

Here you can activate or deactivate the profiles for each color channel for an RGB image. Gray: shows the average intensity value for all three color channels. This function is only available for RGB color images.

X/Y Axis section

Only visible if the **Show All** mode is activated.

Here you can determine the limits for the **X axis** and **Y axis**.

Parameter	Description
Auto	Sets the limits for the axes automatically based on the available pixel values in the image..
Norm	Normalizes the profile display to the maximum values of the distribution.
Fixed	Enter the min/max values for the profile display in the **Min**/**Max** input fields manually.
Log X Scale	Switches the X-axis to logarithmic value distribution.
Log Y Scale	Switches the Y-axis to logarithmic value distribution.

Data Table section

Click on the **Create** button to create a data table of the Profile measurement table. If an interactive measurement value table contains any values, it is also created as separate data table. To save the table as comma separated values file (csv), click on the **Save As** button.

New Image from section

Here you can create an image document of the profile view. Select the desired image configuration from the dropdown list. To save the image, click on the **Save As** button.

13.5.1.5 Histo View

The **Histo** (Histogram) view shows you the gray value histogram of your image. In the right image area you can see your current image and in the left image area you can see the **Histogram window**. At the side you will also find four **data tables**:

- In the first table from the left you will find all the **raw data** for each channel.
- In the second table from the left you will find all the **limits** for each channel of the image next to the image name.
- In the third table from the left you will find the **statistical values** for the gray value distribution, e.g. average, standard deviation, minimum and maximum value.
- The fourth table shows the values of measurements in the histogram. The results (Integral) show the percentaged fractions of the occurences.

See also

General View Options [▸ 887]

13.5.1.5.1 Histo Definition Tab

Tools

With these tools you can add specific ranges to your image. The histogram window displays the gray value histogram for each area.

Parameter	Description
Select	Changes the mouse pointer to Selection mode. You can use this to select graphic elements in the image.
Clone	Use this to copy the last selected element and insert it at another position in the image.
Draw Rectangle	Use this to insert a rectangular measurement region.
Draw Circle	Use this to insert a circular measurement region.
Draw Spline Contour	Use this to insert a measurement region with a shape of your choice. The line is closed automatically.
Draw Polygon	Use this to insert a polygonal measurement region in the original image.
Keep Tool	**Activated:** Keeps the last selected tool active.
Auto color	Only visible if the **Show All** mode is activated. **Activated:** Highlights each drawn-in measurement region with a random color.
Normal	Switches the Profile window back to the view display.
CaliperX	Use this to perform a measurement of a region in X direction in the histogram display. To adopt the value into the measurement data table, click on the **Add to Table** button.
Add to Table	Only active if a measurement (using CaliperX mode) was drawn into the histogram. Adds the current measurement into a measurement data table below the original image.
Reset Table	Deletes the measurement data table below the original image.

Histo Table section

The following parameters are only visible if the **Show All** mode is activated:

Parameter	Description
Histo Table	Select the type of gray value distribution from the dropdown list. The following types are available:
- Frequency	If selected, the histogram is displayed according to the relative frequency of the gray values in percent.

Parameter	Description
- SumUp	
- SumDown	
Relative Frequency	

Parameter	Description
Bin count	Enter the Bin count using the slider.
Bin size	Only visible if the **Show All** mode is activated. Enter the Bin size using the slider.
Logarithmic binning	Only visible if the **Show All** mode is activated. **Activated:** Switches from the linear to a logarithmic class width. The class size is calculated automatically.
Lower Threshold	Enter the lower threshold value for the gray value distribution using the slider or spin box/input field. All regions in the image with gray values below the lower threshold value are overlaid in blue and all those with gray values above the upper threshold value are overlaid in red.
Skip Black	**Activated:** Automatically subtracts the lowest value of the gray distribution. If activated, the settings for the lower threshold value are deactivated.
Upper Threshold	Enter the upper threshold value for the gray value distribution using the slider or spin box/input field. All regions in the image with gray values below the lower threshold value are overlaid in blue and all those with gray values above the upper threshold value are overlaid in red.
Skip White	**Activated:** Automatically subtracts the highest value of the gray distribution. The settings for the upper threshold value are deactivated.
Show thresholds	Only visible if the **Show All** mode is activated. **Activated:** Shows the threshold values as colored overlays in the original image.

13.5.1.5.2 Histo View Tab

Here you can configure the display for the Histo view.

Show section

Parameter	Description
Statistic table	**Activated:** Shows the table containing the statistical values in the image area.
Int. Measurement	**Activated:** Shows the measurement data table below the original image.

Parameter	Description
Frequency Table	Only visible if the **Show All** mode is activated. **Activated:** Shows the table containing the raw data for each channel.
Image	Only visible if the **Show All** mode is activated. **Activated:** Shows the original image in the image area.

Channel section

Here you can activate or deactivate the histograms for each channel.

X/Y Axis section

Only visible if the **Show All** mode is activated.

Here you can determine the limits for the **X axis** and **Y axis**.

Parameter	Description
Auto	Sets the limits for the axes automatically.
Norm	Normalizes the histogram display to the maximum values of the distribution.
Fixed	Enter the min/max values for the histogram display in the **Min**/**Max** input fields.
Logarithmic	

Channel Transparency section

Parameter	Description
Channel Transparency	

Data Table section

Click on the **Create** button to create a data table from all the measured values displayed. To save the table, click on the **Save As** button.

New Image From section

Here you can create a new image document. Select the type of image from the dropdown list. To save the image, click on the **Save As** button.

13.5.1.6 Measure View

In this view measured values from images are displayed in a table. The table is only visible if there are annotations/measured values in the image. To highlight the row of the table containing the measured values of a graphic element, click on a graphic element in the image. To highlight a graphic element in the image, click on the measured value in the row of the table.

13.5.1.6.1 Measurement Tab

Here you can specify how to draw the graphic elements for measurements into an image and how the measurement data are displayed. You can also add user-specific features to individual graphic elements.

Graphic Elements Section

Only visible if the current image is a multidimensional image.

Here you can decide, whether to draw a graphic element "globally" into all channels, Z-positions, time points, etc., or whether to draw in separate elements for the view currently displayed.

Parameter	Description
Channel	**Activated:** Activates the Single Channel mode. Only draws graphic elements into the channel currently displayed.
Time	**Activated:** Only draws graphic elements into the time point currently displayed.
Z-Position	**Activated:** Only draws graphic elements into the Z-position currently displayed.
Copy in All Following	**Activated:** Draws a new graphic element into the view currently displayed and into all subsequent time points or Z-positions.

New Feature section

Here you can add a defined feature to the selected graphic element.

Parameter	Description
Name	Here you can enter a name for the feature.
Value	Here you can enter the desired value for the current graphic element.
Unit	Here you can enter the desired unit for the feature.
Add	Adds the feature. The measurement data table is expanded to include this feature.
Remove	Removes the selected feature.

Data Display section

Here you can specify how you want the measured values for the drawn-in graphic elements to be displayed.

Parameter	Description
Format	
- Table	Displays the measured values in a row of a table. As you can specify the features individually for each graphic element, the number of columns containing measured values may differ from graphic element to graphic element (i.e. from row to row).

Parameter	Description
- List	Displays each measured value in a separate row. The measurement data table then has the following defined columns: ▪ **Name:** Name of the graphic element (e.g. line). ▪ **Feature:** Name of the feature (e.g. distance). ▪ **Value:** Value of the feature. ▪ **Unit:** Unit of the feature (e.g. µm).
Current View	Only displays the measured values of the current view.
All Views	Displays all measured values contained in the image.
Create Document	Creates a measurement data table from the measured values displayed. The table is saved as a separate document.
Export Temperature Data	Only visible for cryo images with temperature data. Opens the temperature data as a table in ZEN.

13.5.1.7 Info View

The **Info View** allows you to display extensive information about your image. Using the buttons in each of the sections you can show additional fields in the sections or hide fields that are currently showing. To show or hide individual sections, click on the button to the left of the headings for each of the sections.

Info

The **Info View** only shows the fields that actually contain data. Using the buttons in each of the sections you can show additional fields. To do this, activate the corresponding checkboxes in the shortcut menu.

13.5.1.7.1 General section

Parameter	Description
Title	Here you can enter a title for your image.
Description	Here you can enter a description for your image.
Comment	Here you can enter a comment.
Keywords	Here you can enter keywords for your image.
Rating	Here you can enter a rating for your image. To enter a rating, click on the star icons.

13.5.1.7.2 File information section

Parameter	Description
Name	Displays the file name of the image without file extension.

Parameter	Description
File Type	Displays the file type of the image.
File Path	Displays the location where the image is saved in your file system.
File Size	Displays the file size of the image.
Created	Displays when the image was created.
Last Modified	Displays when the image was last changed.
User	Displays the name of the user. You can enter the user name in the **Extras** menu | **Options** | *User [▸ 640]*.
Compression Method	Displays the compression method.
Compression Quality	Displays the compression quality.

13.5.1.7.3 Image Dimensions section

Parameter	Description
Time Series	Displays how many time points the image contains. The value in brackets shows the full duration of acquisition.
Z-Stack	Displays how many Z-planes the image contains. The value in brackets shows the full size of the Z-stack.
Channels	Displays how many channels the image contains.
Tiles	Displays how many individual images (tiles) the image is composed of.
Scaling (per Pixel)	Displays the scaling per pixel. **Edit** button: Opens the *Edit Scaling dialog [▸ 827]*.
Image Size (Pixels)	Displays the image size in pixels. The first number indicates the horizontal dimension and the second the vertical dimension.
Image Size (Scaled)	Displays the scaled image size. The first number indicates the horizontal dimension and the second the vertical dimension.
Bit Depth	Displays the bit depth of the active image, e.g. 24 Bit. The bit depth depends on the camera settings when acquiring the image.
Stage Position	Displays the stage position. Within the image this is the center point. In the case of tile images this is the center point of the first tile.
Scanning Mode	Displays the scanning mode. This can either be the image field, an image line or a pixel.
Scanner Zoom	Displays the zoom factor. The value 1 corresponds to the standardized image field of all confocal systems.
Rotation	Displays the rotation of the image field around the optical axis.
Crop Offset	Displays the shift of the scanned region from the center of the image.

Parameter	Description
Pixel Time	Displays for how long the emission signal is collected per pixel. This is the so-called integration time.
Line Time	Displays how long the system needs to scan an image line.
Frame Time	Displays how long the system needs to scan the image field displayed in X and Y in full.
Averaging	Displays the number of individual measurements per image or line. The average of the individual measurements produces the pixel intensity values for the image.

13.5.1.7.3.1 Edit Scaling Dialog

The **Edit Scaling** dialog is divided up into table form. The columns contain the **Scaling Factor** and **Scaling Unit** and the rows the dimensions.

Parameter	Description
Scale Factor column	Enter the desired scaling factor in the input fields.
Scale Unit column	Select the desired scaling unit from the dropdown list. The metric units **Meter**, **Centimeter**, **Millimeter**, **Micrometer**, **Nanometer** and **Picometers** are available as options, as well as the imperial units **Inch** and **Mil**.
Row X	Shows the scaling in the horizontal direction.
Row Y	Shows the scaling in the vertical direction.
Row Z	Shows the scaling in the 3rd dimension. This is usually the focus direction.

Info

Row **Z** for the third dimension is only displayed if the image has a third dimension.

13.5.1.7.4 Acquisition Information section

Parameter	Description
Acquisition Start	Displays the date and time when the acquisition of the image took place.
Microscope	Displays which microscope was used to acquire the image.
Objective	Displays which objective was used to acquire the image.
Optovar	Displays which Optovar was used to acquire the image.
Zoom	
Total Magnification (Eyepiece)	
Filters	

Parameter	Description
Beam Splitter	Displays which beam splitter was used to acquire the channel.
Lasers	
Calibration Marker Posi-tions	
Aperture size	
Magnification	
Accelerating Voltage	
Working Distance	
Scan Speed	
Probe Current	
Specimen Current	
Brightness	
Contrast	
Noise reduction	
Tracks	
Reflector	Displays which reflector cube was used to acquire the image.
Beam Splitter	
Filter Ex. Wavelength	
Filter Em. Wavelength	
Contrast Method	Displays the contrast technique. In transmitted light this is the condenser setting, while in reflected light it corresponds to the selected reflector cube.
Ligth Source	
Ligth Source Intensity	Displays the lamp intensity with which the image was acquired.
Illumination Wavelength	
Pinhole	Displays the diameter of the pinhole.
Laser Wavelength	
Laser Blanking	Blanking of the laser during scanner movement without acquisi-tion.
Stokes Vector	
Laser Attenuator State	
Scan Mode	
Rotation	
Crop offset	

Parameter	Description
Pixel Time	
Line Time	
Frame Time	
LSM Scan Speed	
Scan Direction	
Line Step	
Averaging	
Averaging Mode	
Averaging Method	
VivaTome Grid	
VivaTome Reflector	
VivaTome Beam Splitter	
VivaTome Filter Ex. Wave-length	
VivaTome Filter Em. Wavelength	
Channels	
Channel Name	Displays the name of the channel.
Channel Description	Here you can enter a description of the channel. Describe the exact use of the channel or what can be seen in this channel.
Dye Name	Displays the name of the dye.
Channel Color	Displays the pseudo color allocated to the channel.
Emission Wavelength	Displays the main emission wavelength of the channel or dye used.
Excitation Wavelength	Displays the main excitation wavelength of the channel or dye used.
Effective NA	
Detection Wavelength	
Imaging Device	
Camera Adapter	Displays which camera adapter was used to acquire the image.
EM Gain	Displays the factor by which the camera signal was increased.
Exposure Time	Displays the exposure time with which the image was acquired.
Depth of Focus	Displays the depth of focus. This is calculated according to the following formula: Depth of field = $(2 * n * \lambda) / (NA)^2$ = (2 * refractive index * emission wavelength) / (numerical aperture)^2

Parameter	Description
Section Thickness	Displays the thickness of the optical section.
Binning Mode	Displays whether binning was applied during acquisition and how much.
Detector Type	Displays which detector was used for acquisition.
Detector Gain	Displays the gain setting of the detector for acquisition.
Detector Digital Gain	Displays the digital gain of the detector during acquisition.
Detector Offset	Displays the offset settings of the detector during acquisition.
HDR Processing	
Airyscan Mode	

Info

In the case of multichannel images the channel-dependent information is saved in a table. Here the sorting of the individual information fields may differ.

13.5.1.7.5 Direct Processing section

This section is only visible if your image was processed with Direct Processing.

Parameter	Description
Processing Function	Displays the name of the function which was used for Direct Processing.
Completed	Displays the date and time when the processing was finished.
Duration	Displays the duration of the processing.

13.5.1.7.6 Deconvolution Information section

This section is only visible if the image was processed with **Deconvolution**. The displayed parameters depend on which algorithm was used.

Parameter	Description
Algorithm	Displays which algorithm was used for processing.
Elapsed Time	Displays how long the processing took.
Aberration Correction	Displays what/if aberration correction was used for processing.
GPU Acceleration	Displays what/if GPU acceleration was used for processing.
Optimization	Displays the optimization which was used for processing.
Quality Threshold	Displays the quality threshold which was used for processing.
Normalization	Displays the normalization method which was used for processing.

Parameter	Description
Strength	Displays the normalization strength which was used for processing.
Stack Correction	Displays what/if stack correction was used for processing.
Regularization	Displays the regularization which was used for processing.
Likelihood	Displays the likelihood calculation which was used for processing.
PSF	Displays the PSF which was used for processing.
-> Clipboard	Saves the parameters to the clipboard. For reuse of parameters for new processing, see also *Step 4: Info View and re-using Deconvolution parameters from a processed image [98]*.
Convergence History	Displays the convergence history graph.

13.5.1.8 Tree View

The **Tree View** is visible only if you have activated the **Enable Tree view** checkbox under **Tools** | **Options** | **Documents**. The checkbox is deactivated by default.

The tree view shows a detailed list containing all meta data of the selected image.

13.5.2 Specific image views

These image views are only visible if the image has corresponding features. The **3D view**, for example, is only visible for Z-stack images.

13.5.2.1 Split View

Only visible for multichannel images and not during the acquisition of LSM images.

In this view you see all channels of a multichannel image. The channels are displayed side by side, in the channel colors that have been assigned to them. You also see the mixed image view in which all the channels are overlaid.

Info

By double-clicking on an acquired multi-channel image, you can switch quickly to the **2D** view. Double-clicking on the image in the **2D** view switches you back to the **Split** view. If you double-click on one of the displayed channels, only this channel will be shown in **2D** View.

See also

- 2D View [810]
- General View Options [887]

13.5.2.2 Ortho View

Only visible for Z-stack images.

In this view (orthogonal section) you can analyze your Z-stack images. Here, in addition to the top view (X/Y axis), you will also see the section views of the X/Z axes (top) and Y/Z axes (right).

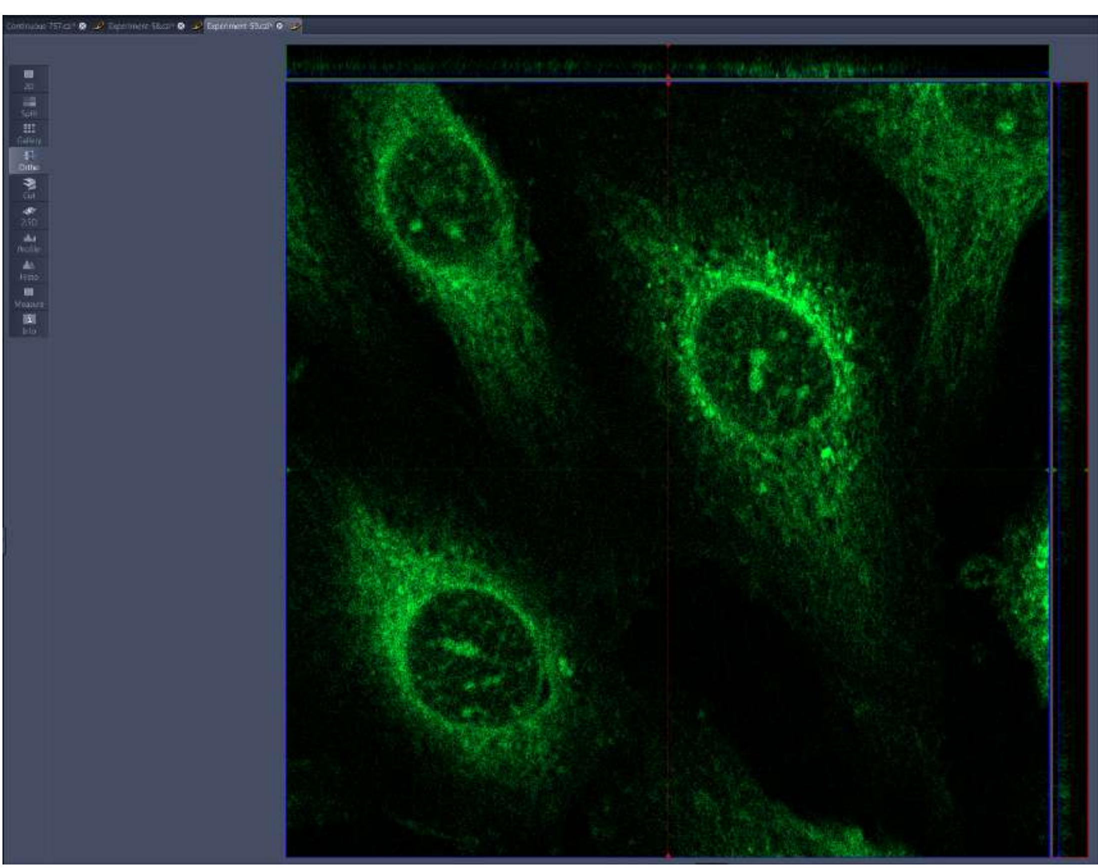
See also

General View Options [887]

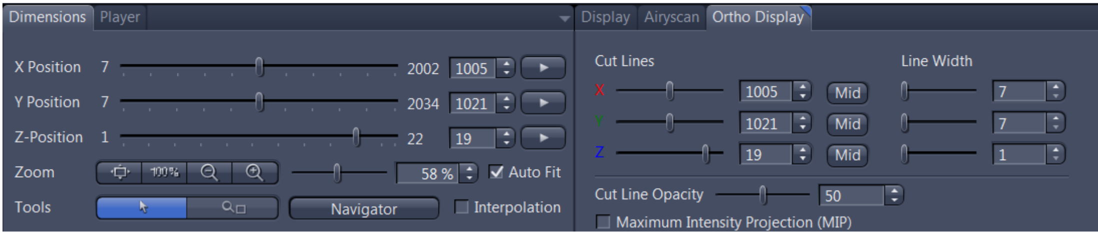
13.5.2.2.1 Ortho view for Airyscan images

Airyscan multiplex images can be directly viewed during acquisition in 3D ortho view.

Select the **Ortho** view tab to switch to this display mode.

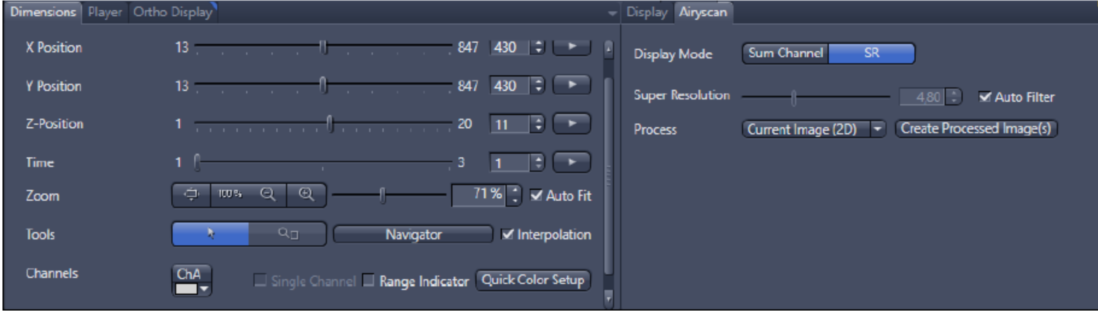


In **Ortho Display**, you can adjust the areas which are sectioned for the 3D view.



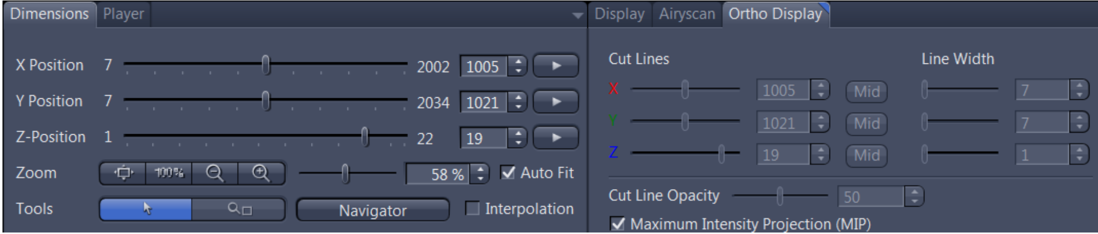
Ortho Display can be combined with Airyscan **SR** preview processing.

To activate the preview, click on the **SR** button in the **Airyscan** tab.

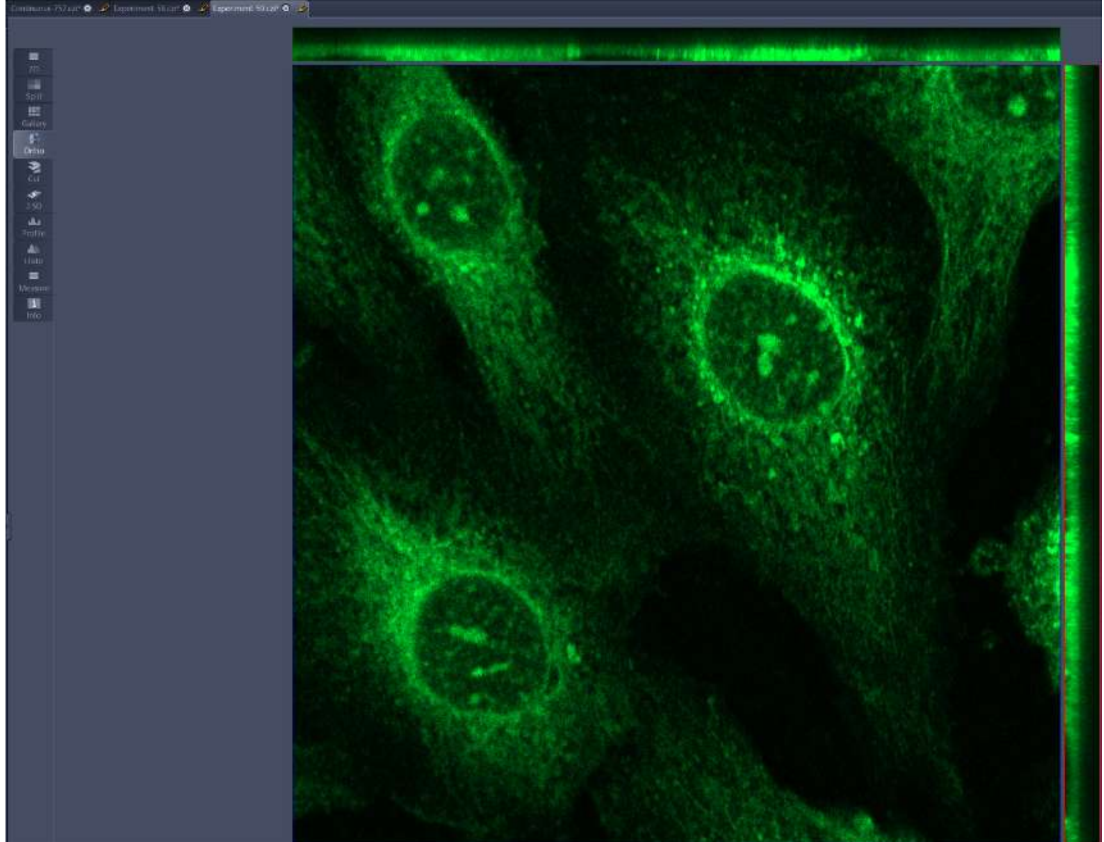


Ortho Display also allows to display a **Maximum Intensity Projection (MIP)** in all 3 directions.

Activate the **Maximum Intensity Projection (MIP)** checkbox to activate this view. It is recommended that only fully acquired stacks are viewed with this function.



The result is a brighter XY image, and a very bright XZ and YZ view. This is because very many slices are projected here. It might also occur that the XZ and YZ view show stripes. This is due to the preview processing and often disappears once the full datastack was processed and saved.



13.5.2.2.2 Ortho Display Tab

Parameter	Description
Cut Lines	Sets the positions (pixel values) for the section lines using the **X/Y/Z** sliders or input fields. Alternatively you can also adjust the positions directly in the image area. To adjust the positions, move the mouse over a section line in the image. Hold down the left mouse button and move the mouse.
- Mid	Positions the relevant slider at the center of the view.
Line Width	Only visible if the **Show All** mode is activated. Enter the thickness of the section lines in pixels using the sliders or input fields. This results in a maximum intensity projection being displayed over the selected pixel width.
Cut Line Opacity	Only visible if the **Show All** mode is activated. Here you can enter the degree of opacity of the section lines from 0% (invisible) to 100% (completely opaque).
Maximum Intensity Projection (MIP)	**Activated:** Displays a maximum intensity projection (MIP) across all planes for all 3 views. The section lines are hidden and the control elements that are not relevant in this view are deactivated.
Measure 3D Distance	Only visible if the **Show All** mode is activated. **Activated:** Activates the 3D distance measurement. The **Set Start** and **Set End** buttons and the **Distance** display field are visible. To set a starting point for the measurement, navigate the cutlines to the desired starting point and click on the **Set Start** button. The **Set End** button will become active. To set an end point for the measurement, navigate the cutlines to the desired end point and click on the **Set End** button. The pixel coordinates of the measurement points are displayed next to the buttons. The measured distance is displayed in the **Distance** display field.
New Image	Creates a new image document. Select the desired view from the dropdown list (only in **Show All** mode). To save the image, click on the **Create** button. The resulting image contains the image data in the same dynamics (bit depth) as the original image and consists of the same number of channels (in the case of multichannel images) or time points (in the case of time lapse images) as the original image, but only contains the Z-plane currently displayed.

13.5.2.3 3D View

This view is only available if:

- you have licensed the **3Dxl** module.
- you have loaded or acquired a z-stack image.
- a suitable NVIDIA or ATI graphics card with full OpenGL 3.3 or higher functionality is present.

3D View employs 3D rendering technology which requires access to advanced Open GL functionality. For full functionality this means, that a modern dedicated graphic card (NVIDIA or AMD-ATI technology) has to be present. The lower grade graphics functionality provided with some Intel GPUs frequently found on laptops, does not support all of the required Open GL calls. This means, that with Intel GPUs, only reduced functionality is available:

- only maximum projection rendering (MIP).
- **Series** tab is missing.
- only reduced functionality of the **Appearance** tab.
- No fonts can be displayed in the render window.
- the **Measure** button opens the measurements table but does not show any measurements.

The 3D view displays z-stack images three-dimensionally as a 3D volume. Using the toolbars on the left, right and bottom of the image area you can directly control and move the 3D volume. With the view specific tabs below the image area you find a lot of parameters to adjust the appearance and further settings of the 3D volume.

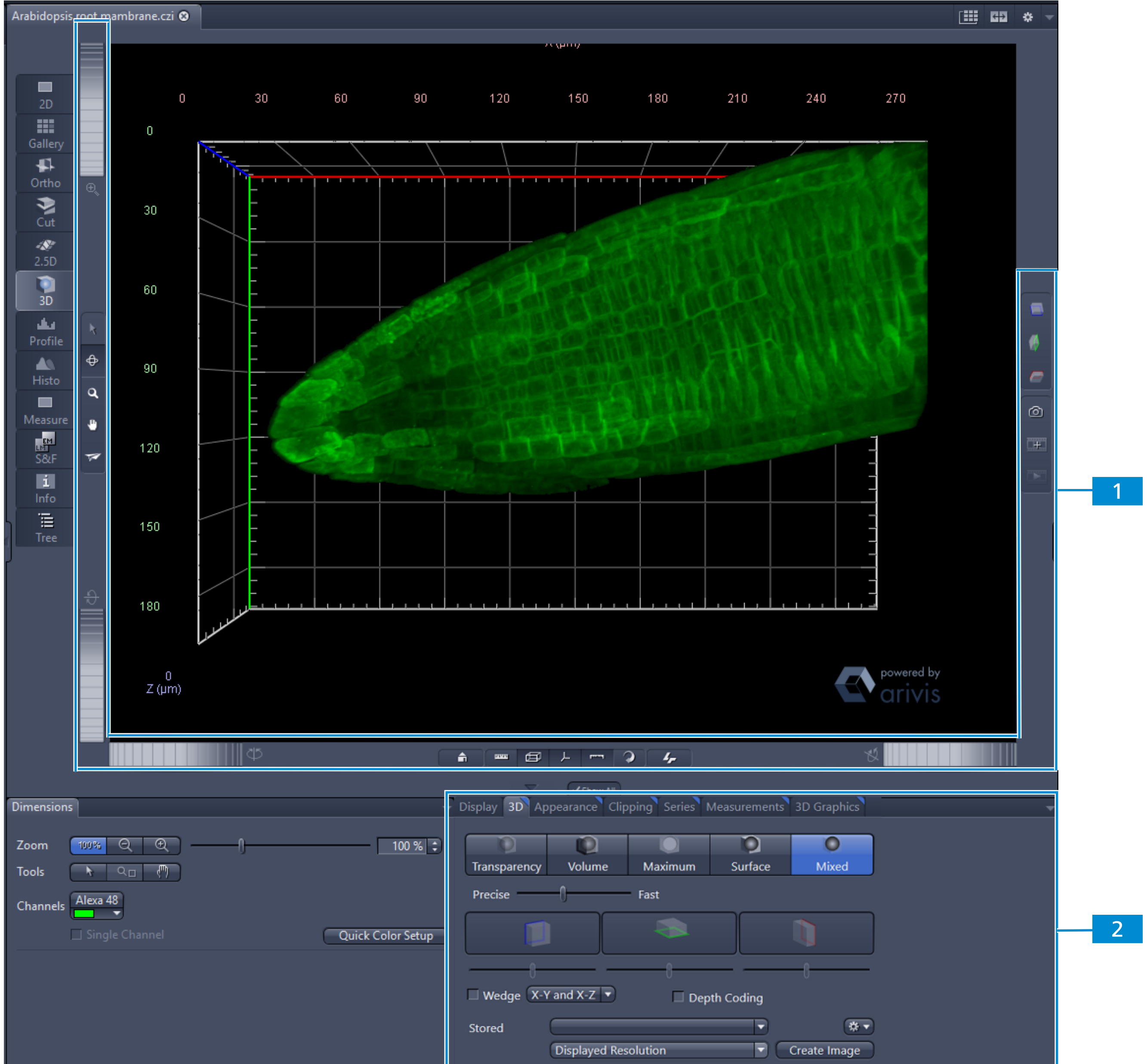


Fig. 71: 3D View

1 **Tool bars**
see *Tool bars [▸ 836]*

2 **View Specific Tabs**
see *3D Tab [▸ 840]*,
Appearance Tab [▸ 841],
Clipping Tab [▸ 843],
Series Tab [▸ 845],
Measurements Tab [▸ 848],
3D Graphics Tab [▸ 849]

13.5.2.3.1 Tool bars

The tool bars are arranged to the left and right of the image area and underneath it. You can use the tools to control and adjust the display of the 3D volumes in the image area. The tool bars are visible in ZEN's standard 3D view as well as the ZEN Connect 3D view. For the view in ZEN Connect not all of the options are available.

13.5.2.3.1.1 Tool Bar (Left)

This tool bar is visible in the ZEN standard 3D view and the ZEN Connect 3D view. Not all the options are available for each view.

Parameter	Description
Top thumb wheel	Zooms in or out of the 3D image.
Arrow	Use this to select end points of measurement tools that have been drawn into the 3d image (**Measure** tab). You can then edit the position of the end points. In the ZEN Connect 3D view, this control allows you to transform the adjustable volume.
Rotate	Use this to rotate the 3D image in any way you wish within the space. This is the default mode when you switch to **3D view** for the first time.
Zoom	Use this to increase or reduce the zoom factor of the image area.
Move	Use this to move the 3D image. In the ZEN Connect 3D view, this control allows you to shift the z-stack in the z-plane.

Parameter	Description
Fly	Clicking on this button enables the flight mode. This mode allows to virtually fly through the 3D image. Use the keys from the list below to control your flight.
Bottom thumb wheel	Rotates the 3d image around the horizontal (X) axis.

Flight Mode Key Layout / Controls

Key	Function
W	Forward
S	Backward
A	Left
D	Right
Space	Up
C	Down
E	Rotate (clockwise)
Q	Rotate (counter-clockwise)
X	Precision Mode, enables slower movement

13.5.2.3.1.2 Tool Bar (Right)

This tool bar is visible in ZEN's standard 3D view as well as the ZEN Connect 3D view. For the view in ZEN Connect not all of the options are available.

Parameter	Description
Toggle X/Y clipping plane (blue)	Hides the X/Y clipping plane.

Parameter	Description
Toggle X/Z clipping plane (green)	Hides the X/Z clipping plane.
Toggle Y/Z clipping plane (red)	Hides the Y/Z clipping plane.
Snap	Creates a 2D image of the current view. The image is a 24 bit color image. All annotations are burnt in automatically.
Add	Adds the current view to a position list as a new position. With the help of position lists you can have your view calculated as a series of individual images. This series can then be exported as a movie, for example.
Play	Only active if a position list containing at least two saved positions exists. Plays back a preview of the series that is calculated. To stop the preview, click on the button again.

13.5.2.3.1.3 Tool Bar (Bottom)

This tool bar is visible in the ZEN standard 3D view and the ZEN Connect 3D view. Not all the options are available for each view.

Control element	Description
Left thumb wheel	Rotates the 3D volume around the vertical (Y) axis.
Home view button	Switches back to the start view from any view. A top view of the 3D volume is displayed. Lateral movements and the zoom factor are adjusted so that the 3D volume can be seen at the center of the image area.
Show measurements button	Shows or hides drawn-in measurements. If measurements are drawn-in, a table of the measurements appears at the right side of the image area.
Show bounding box button	Shows or hides a bounding box around the 3D volume.

Control element	Description
Show coordinate axes in color button	Shows or hides the coordinate axes. ▪ X axis = red ▪ Y axis = green ▪ Z axis = blue
Show scaling button	Shows or hides the scaling on each axis.
Spin Mode button	Enables the spin mode. This allows to set the 3D volume in continuous motion. You will find a short description on how to use the spin mode below.
Send to Vision 4D	Only active, if **arivis Vision 4D** is installed and licensed on the system. Starts **Vision 4D** and imports the image. The image will be displayed with almost identical rendering settings as in **ZEN**'s **3D View**. **Note:** Not all render methods will produce absolutely identical settings between the two applications. This is due to additional functionalities available for **Vision 4D**.
Apply	Only available in Zen Connect 3D view. Applies the changes made in the 3D view to the ZEN Connect project.
Reset	Only available in Zen Connect 3D view. Restores the settings defined in the ZEN Connect project.
Right thumb wheel	Rotates the 3D volume around the (Z) axis perpendicular to the screen plane.

13.5.2.3.1.4 Animating the 3D Volume

Prerequisite ✓ The **Rotation** mode in the left tool bar is selected.

1. Click on the **Spin mode** icon.

2. Move the mouse inside the image area.
3. Hold down the left mouse button and move the mouse slightly to the left or right.
4. Release the left mouse button again.

The 3D volume rotates continuously in the direction in which you moved the mouse. If you move the mouse quickly, the 3D volume rotates quickly. If you move the mouse slowly, the 3D volume rotates slowly.

To stop the animation, left-click again in the image area.

13.5.2.3.2 3D Tab

Here you can specify which projection/rendering mode you want to use to display the 3D volume. There are 5 view modes available. To activate the desired view mode, click on the corresponding button. An activated button (respectively the mode) appears in blue color.
Note: This tab is visible in the ZEN standard 3D view and the ZEN Connect 3D view. Not all the options are available for each view.

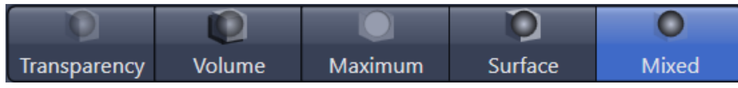


Fig. 72: Mode Buttons

Parameter	Description
Transparency	Activates the Transparency rendering mode.
Volume	Activates the Volume mode.
Maximum	Activates the Maximum intensity projection mode.
Surface	Activates the Surface reconstruction mode
Mixed	Activates a combination of transparency rendering and surface reconstruction mode
Precise/Fast	Adjust the level of detail of the 3d volume here. ▪ If you select the **Precise** setting, all the information present in the image is used to achieve the best possible display. The calculation time can increase accordingly. ▪ If you select the **Fast** setting, the image data are significantly reduced before the calculation. The calculation is fast, but only a very coarse 3D display of the volume is achieved.
Update on completed frame	Only available, if **Follow Acquisition** on the **Dimensions** tab is activated. **Activated:** During acquisition, the 3D View is only updated after the acquisition of a stack has been completed. **Deactivated:** While the acquisition of a stack is still ongoing, the 3D View is continuously updated. However, with very fast acquisition this update can not be guaranteed.
Depth coding	Only active if **Transparency**, **Maximum** or **Mixed** mode is activated. **Activated:** Replaces the channel colors of the volume with a rainbow color table where the color represents the depth of the stack. A palette is shown which matches the colors to a given depth (in scaled units).
Toggle Clipping Planes	By activating or deactivating the buttons you can show or hide the corresponding clipping planes in the 3D volume. If you right-click on an activated button, a shortcut menu opens. Here you can select whether you want the back (**Clip Back**), front (**Clip Front**) or both sides of the 3D volume to be clipped. You can also specify the **Style** of the clipping plane. Under each button is a slider. You can use this to move the relevant clipping plane within the volume.

Parameter	Description
Wedge	**Activated:** Activates two texture planes. Only the sector between the planes is cut out. You can select which planes you want to be used for the wedge function from the dropdown list. The selection is also visible in the relevant buttons.
Stored	Here you can select saved 3D settings.
Options	Opens the options menu.
– New	Creates a new settings file that is given a name automatically and has the file extension *.cz3dr. The settings file can be found in the user path under \My Documents\Carl Zeiss\ZEN\Documents\3Dxl render settings.
– Delete	Deletes the selected settings file from the hard drive.
– Rename	Renames the selected settings file. Enter a new name in the input field and confirm with **OK**.
– Save As	Saves the selected settings file under a different name.
– Import	Imports a ***.cz3dr** file and applies it to the current image.
– Export	Exports a ***.cz3dr** file to a different location.
Create Image	Creates a new image from the current view. This image is a 24 bit RGB color image. All graphic elements, such as annotations, are burnt in. In the dropdown left of the button you can select the resolution for the image that is created.

13.5.2.3.3 Appearance Tab

Here you can define the appearance of the 3D volume. On the tabs available on this tab, select the setting that you want to change (e.g. Transparency). Depending on which mode you have activated on **3D** tab, different tabs and parameters are available.
Note: This tab is visible in the ZEN standard 3D view and the ZEN Connect 3D view. Not all the options are available for each view.

13.5.2.3.3.1 Transparency Tab

Parameter	Description
Alexa Fluor 488 All **Channel selection**	Here you can select the channel of a multichannel image for which you want to set the transparency.
Threshold	Sets the lower threshold value in percent of the gray levels displayed. With this setting you specify the gray value range for the relevant channel that you want to be included in the rendered image.
Ramp	Sets the extent of the transition from completely transparent to completely opaque (0-100 percent).
Maximum	Sets the level of opacity (0-100 percent).

Parameter	Description
Histogram	Displays the settings that you enter using the sliders schematically. The X axis represents the gray level values and the Y axis the opacity. You can also change the position of the curve using the mouse.
Reset	Resets all parameters to the original values.

13.5.2.3.3.2 Surface Tab

Only visible if **Surface** or **Mixed** view mode is activated on the **3D** tab.

Parameter	Description
Channel selection	Here you can select the channel of a multichannel image for which you want to adjust the surface settings.
Threshold	Sets the lower threshold value in percent of the gray levels displayed. With this setting you specify the gray value range for the relevant channel that you want to be included in the rendered image.
Ambient Light	Sets the ambient light on a scale from 0 to 100%.
Spectacular Light	Sets the spectacular light from 0 to 100%. This value influences the differences between bright and dark structures.
Shininess	Sets the surface shininess.
Reset	Resets all parameters to the original values.

13.5.2.3.3.3 Channels Tab

Only visible if **Mixed** view mode is activated on the **3D** tab.

Here you can specify how **Transparency** and **Surface** settings are mixed. In the case of multi-channel images you can also configure these settings differently for each channel.

Activate the corresponding checkboxes for Transparency and Surface in the list.

13.5.2.3.3.4 Background Tab

Parameter	Description
Background Color	Sets the background color for the 3D view. To do this, click on the color field and select the desired color.
Reset	Resets the background color.

13.5.2.3.3.5 Light Tab

Parameter	Description
Brightness	Sets the brightness of the light source (from 0 - 100 %).

Parameter	Description
Azimuth	Here you can enter the angle of the light source above the virtual horizon.
Elongation	Here you can enter the light source's horizontal angle of incidence.
Light source	As an alternative to the slider or input field, you can set the **Azimuth** and **Elongation** together by using the mouse to move the point within the light source display.
Reset	Resets all parameters to the original values.

13.5.2.3.3.6 Projection Tab

Parameter	Description
View angle	Sets the projection angle at which you want to view the scene freely between 0° and 80°. The effect of this on the perspective display is as if you are viewing the 3D image through a telephoto or wide-angle lens.
Scale Z	Here you can set the scaling of the volume in the Z direction (value range 10% - 600%).
Stereo anaglyph	**Activated**: Displays the 3D volume as anaglyphs. You can choose between a ▪ **Red/Green** display, or a ▪ **Red/Cyan** display.
Camera separation	Sets the distance between the two virtual cameras (0-20%).
Parallax shift	Sets the degree of movement that is necessary to bring the two camera images back into line (-100 to +100%).
Reset	Resets all parameters to the original values.

13.5.2.3.4 Clipping Tab

Only visible if the **Show All** mode is activated.

Here you can edit clipping planes. To select a clipping plane, click on the corresponding button. The editing functions which you can use to modify the selected clipping plane become visible when you activate the specific clipping plane.

Info

On the **Clipping** tab you can edit the clipping planes. On the **3D** tab you can activate or deactivate the relevant clipping planes in the 3D volume.

Parameter	Description
Show All Clipping Planes	**Activated:** Automatically inserts all 3 clipping planes into the 3D volume. Additionally the editing functions for each clipping plane were activated automatically.

Parameter	Description
X/Y	Activates the editing functions for the X/Y clipping plane.
X/Z	Activates the editing functions for the X/Z clipping plane.
Y/Z	Activates the editing functions for the Y/Z clipping plane.
Activate	**Activated:** Activates the selected clipping plane in the 3D volume. The corresponding settings become visible. You will find a detailed description of the settings in the list below. **Note:** Each plane is positioned at the center of the 3D volume and aligned orthogonally (in the X/Y, X/Z, Y/Z direction).
Reset All	Resets all parameters to the original values.

The following parameters are only visible if the **Activate** checkbox is activated and a clipping plane has been selected.

Parameter	Description
Clipping Plane Style	Change the display of the selected clipping plane using the dropdown list to the right of the **Activate** checkbox. The following settings are available:
- Invisible	The plane is invisible.
- Colored	The plane is displayed in color. The frame color is used with 50% transparency here.
- Binary	The data above the threshold value that are touched by the clipping plane are displayed in binary form as a white area. Black pixels are non-transparent.
- Transparent	The data that are touched by the clipping plane are displayed as they are in **Transparent** view mode, but in 2 dimensions. The ramp for the transparency is linear here. Black pixels are transparent.
- Textured opaque	The display appears as it does with the **Textured** setting. Black pixels do not let any light through, however, meaning that the render data behind them are not displayed.
Outline	**Activated:** Displays the frame of the selected clipping plane. Enter the frame color via the color field.
Clip Front	**Activated:** Clips the front of the 3D volume.
Clip Back	**Activated:** Clips the back of the 3D volume.

Parameter	Description
Clip Transparency	Only active if **Mixed** view mode is activated. **Activated:** In addition to the surface data, also clips the transparency data.
Clip Surface Channels	Only visible if **Surface** or **Mixed** view mode is activated. Here you can enter which channel you want to be clipped using the channel buttons.
Position	Here you can enter the position of the selected clipping plane.
<X (X Angle)	Here you can enter the X angle for the selected clipping plane.
<Y (Y Angle)	Here you can enter the Y angle for the selected clipping plane.
Reset Orientation	Resets the selected clipping plane to the original position.

13.5.2.3.5 Series Tab

Here you can create render series of individual views, which you can later view and export as a movie. The tab contains different control elements depending on the Render Series type. The following parameters are the same for all render series types: **Render Series** section, **Stored** section, **Apply** button and **Fixed Resolution** checkbox.
Note: This tab is available in the ZEN 3D view as well as in the ZEN Connect 3D view. Not all the options and settings are available in the ZEN Connect 3D view!

Parameter	Description
Render Series	Here you can select the desired series mode. Depending on the chosen render series type, different parameters are displayed.
- Turn Around X	Define the start/stop angle and the rotation direction around the X axis.
- Turn Around Y	Only visible in the **3D** view. Define the start/stop angle and the rotation direction around the Y axis.
- Turn Around Z	Define the start/stop angle and the rotation direction around the Z axis.
- Start/Stop	Define the angle and zoom settings for the start and end position of your series. The intermediate positions are interpolated evenly.
- Position list	Define any number of positions. The positions can each have completely different rotation, zoom and illumination settings.
- Over Time	Only visible in the **2.5D** view. Define the start time point and end time point for a series. All other settings (rotation, zoom, etc.) remain unchanged.
Apply	If clicking on this button the series will be calculated. A new image document will be opened in the **Center Screen Area**. You can view the series by clicking on the **Play** button in the **Dimensions** tab.

Parameter	Description
Stored	Only visible if **Show All** is activated. Here you manage your series settings. Via the dropdown list you can select a saved settings file.
Options	Clicking on the button opens a shortcut menu with the following options:
- New	Creates a new settings file (***.czsht**). This file can be found in the user's local document path (e.g. \My Documents\Carl Zeiss\ZEN\Documents, in a corresponding subfolder).
- Delete	Deletes the selected settings file.
- Rename	Opens a dialog to rename the selected settings file.
- Save As	Saves a copy of the currently selected settings file under a different name.
- Save	Saves changes to a currently selected settings file.
- Import	Imports a settings files from the hard disk.
- Export	Exports a settings files to the hard disk.
Preview	Shows a preview of the series to be created. Use the **Play** / **Stop** button to start or stop the preview.
Frames	Sets the number of individual frames that the series consist of after the calculation. The more individual images that you specify here, the more fluidly the scene transitions will be displayed later. Select predefined values from the dropdown list (e.g. **20** or **100** frames).
Fixed Resolution	Only visible if **Show All** is activated. As a rule, the image series is calculated using the current screen resolution. If you want to set a different format for the series, activate the checkbox. In the input fields that are now visible you can enter the width and height in pixels with which you want to series to be created.

The following parameters are only available if you have selected **Turn Around X / Y / Z** under **Render Series**:

Info

- The X rotation, Y rotation and Z render series types all have the same control elements and differ only in the axis around which the rotation is calculated.
- The preview function is not available for these types of series.

Parameter	Description
360° Panorama	Select 360° panorama, if you want to generate a complete rotation series.
Partial Panorama	If you select partial panorama, you can specify the starting angle and stopping angle that you want to use for the series. To do this, enter the desired values in the input fields or adjust it in the graphical representation of the rotation circle at the right of the input fields.
- Start Angle	Determines the starting angle.
- Stop Angle	Determines the stop angle.
- Direction	Determines the direction of rotation.
- Angle Definition	When you are configuring a partial panorama, the desired angles can also be determined easily using the circular control element: Grab the white start/stop points with the mouse and position these accordingly on the circle. The number of individual images is also displayed here.

The following parameters are only available if you have selected **Start/Stop** under **Render Series**:

Parameter	Description
Start Position	You can position the volume in the image area as required using the mouse. The geometric parameters are displayed in the input fields. You can also determine the **Camera Position** and the **Look At** parameters for X, Y or Z and the angle directly using the input fields or the slider. All changes are displayed immediately in the image area.
Stop Position	You can position the volume in the image area as required using the mouse. The geometric parameters are displayed in the input fields. You can also determine the **Camera Position** and the **Look At** parameters for X, Y or Z and the angle directly using the input fields or the slider. All changes are displayed immediately in the image area.

The following parameters are only available if you have selected **Position list** under **Render Series**:

Parameter	Description
Add	Adds the current position to the position list.
Insert	Inserts a new position between two existing positions.

Parameter	Description
Position list	Each position is displayed in the list with its X, Y, Z angle and zoom level. Using the control elements at the bottom of the list you can change the order of the positions (**Arrow** buttons), cut positions (**Scissors** icon) or copy and paste them again at another position (**Copy / Paste** icons). If you want to delete all positions, click on the **Clear List** button.
Further Parameters	You can determine which of the following parameters you want to be taken into consideration when the series is calculated. To do this, activate the corresponding checkbox:
- Light	Includes illumination parameters.
- Transparency	Includes transparency settings (not active in **Surface** mode).
- Background	Includes color and distance of the background.
- Time	Includes time series parameters (only active for time series images).
- Camera	Includes camera settings, e.g. viewing angle (from the 3D / virtual camera).
- Planes	Includes planes settings (not active in **Shadow** mode).
- Surface	Includes surface settings (only active in **Surface** and **Mixed** mode).

13.5.2.3.6 Measurements Tab

Only visible if the **Show All** mode is activated.

Here you can perform interactive measurements in the 3D volume. Note that measurements are not possible in **Shadow** projection mode. The measurements can be drawn in directly in the 3D volume using different tools. The measurement results are displayed in a list at the right of the image area.

Parameter	Description
Tool bar	Using the tools you can perform interactive measurements in the 3D volume. The following tools are available:
- Select	Changes the mouse pointer to Selection mode. Use this to select measurements in the 3D volume in order to change them.

Parameter	Description
- Line	Use this to measure the length of a line in µm. Click once on the starting point and hold down the mouse button. Then drag the mouse to the end point and release the mouse button again. The measurement is complete. The result of the measurement is displayed in the list to the right of the image area.
- Angle	Use this to measure the angle between two connected legs. First define the starting point. Then use the mouse to drag the first leg to the desired first end point. Define the second leg by clicking on the second end point. The angle measurement ends with a display of the angle measured (in degrees). The result of the measurement is displayed in the list to the right of the image area.
- Polygon Curve	Use this to measure along a line with any number of segments. Click from corner point to corner point. Complete the measurement by right-clicking. The result of the measurement is displayed in the list to the right of the image area.
- Color selection	Here you can select a color for the tool you want to draw in. Simply click on the colored rectangle and choose a color from the list.
- Keep Tool	**Activated:** Keeps the selected tool active.
- Auto Color	**Activated:** Automatically changes the color of the drawn-in tool.

Parameter	Description
Show Measurements	**Activated:** Shows the measurements in the 3D volume or in the list of measured values at the right of the image area.
- On top	**Activated**: All drawn-in measurement tools appear in the foreground, even if these are in fact obscured by image structures.
Display Values	
- on the objects	**Activated:** Displays the measured values in the 3D volume.
- as list	**Activated:** Displays the measured values in the measurement data table.
Delete Selected	Only active if a measurement tool has been selected in the 3D volume. Deletes selected measurement tools from the 3D volume.
Delete All	Deletes all measurement tools from the 3D volume.

13.5.2.3.7 3D Graphics Tab

Only visible if the **Show All** mode is activated.

Parameter	Description
Tool bar	Keep Tool Auto color Using the tools you can perform interactive measurements in the 3D volume. The following tools are available:

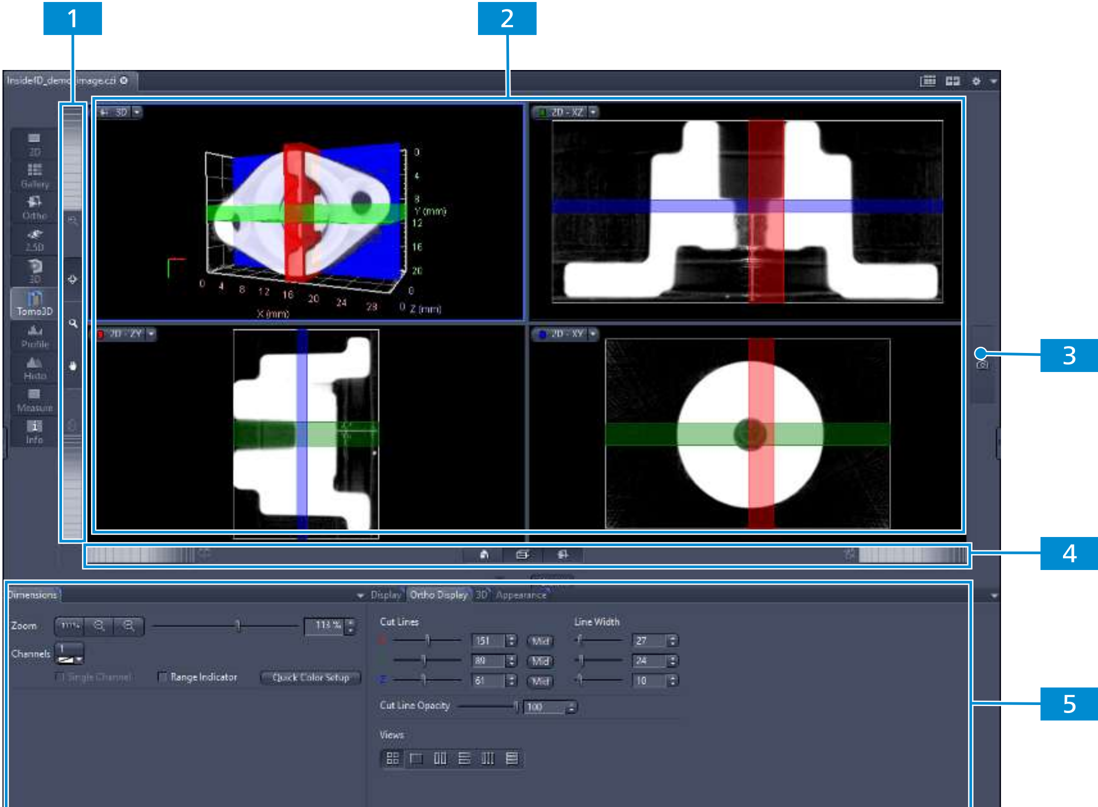
Parameter	Description
- Select	Changes the mouse pointer to Selection mode. Use this to select measurements in the 3D volume in order to change them.
- Line	Use this to measure the length of a line in µm. Click once on the starting point and hold down the mouse button. Then drag the mouse to the end point and release the mouse button again. The measurement is complete. The result of the measurement is displayed in the list to the right of the image area.
- Angle	Use this to measure the angle between two connected legs. First define the starting point. Then use the mouse to drag the first leg to the desired first end point. Define the second leg by clicking on the second end point. The angle measurement ends with a display of the angle measured (in degrees). The result of the measurement is displayed in the list to the right of the image area.
- Polygon Curve	Use this to measure along a line with any number of segments. Click from corner point to corner point. Complete the measurement by right-clicking. The result of the measurement is displayed in the list to the right of the image area.
- Color selection	Here you can select a color for the tool you want to draw in. Simply click on the colored rectangle and choose a color from the list.
- Keep Tool	**Activated:** Keeps the selected tool active.
- Auto Color	**Activated:** Automatically changes the color of the drawn-in tool.

Parameter	Description
3D Measurements	All measurement contained in the 3D volume are displayed here. The list contains the following columns:
- Eye icon	Here you can select whether or not a measurement tool is displayed in the image. If you click in the title field of the column, the setting is made simultaneously for all entries.
- Lock icon	Not activated for the 3d view.
- Type	Displays the type of a tool. If you click on the icon, you can change the color of the tool.
- ID	Displays the unique identification number of the measurement tool.
- A	No function
- M	**Activated:** Displays the measurement data in the image. If you click in the title field of the column, the setting is made simultaneously for all entries.
- Name	Displays the name of the tool. To change the name, double-click on the entry. Then enter a new name. Confirm the entry with the Enter key.

13.5.2.4 Tomo3D View

The Tomo3D view combines a 3D viewer with up to three orthogonal 2D views.
The Tomo3D is only available if:

- you have the **3Dxl Plus** license.
- you have loaded or acquired a z-stack.



1 Left Toolbar
Toolbar to manipulate the image display. For more information, see *Left Toolbar (Tomo3D) [▸ 852]*.

2 Image View
Area where you interact with the image views and set the cut lines with the mouse. You can display up to four different views, including a 3D and different 2D views. The number of image views can be set in the **Ortho Display** tab, the type can be set by the dropdown in the top left corner of each view.

3 Snap button
Creates a 2D image of the current view. All annotations are burned in automatically.

4 Bottom Toolbar
Toolbar to manipulate the image display. For more information, see *Bottom Toolbar (Tomo3D) [▸ 852]*.

5 View Options
Area for general view options, like the *Dimensions Tab [▸ 887]*, and the specific view options.

13.5.2.4.1 Left Toolbar (Tomo3D)

Parameter	Description
Top thumb wheel	Zooms in or out of the image.
Rotate	Use this to rotate the 3D image in any way you wish within the space.
Zoom	Use this to increase or reduce the zoom factor of the image area.
Move	Use this to move the 3D image.
Bottom thumb wheel	Rotates the 3D image around the horizontal (X) axis.

13.5.2.4.2 Bottom Toolbar (Tomo3D)

Control element	Description
Left thumb wheel	Rotates the 3D volume around the vertical (Y) axis.
Home view button	Switches back to the start view from any view. A top view of the 3D volume is displayed. Lateral movements and the zoom factor are adjusted so that the 3D volume can be seen at the center of the image area.

Control element	Description
Show bounding box button	Shows or hides a bounding box around the 3D volume.
Draw cutlines	**Activated:** Displays the cutlines in the 3D viewer. **Deactivated:** The cutlines are not displayed in the 3D viewer.
Right thumb wheel	Rotates the 3D volume around the (Z) axis perpendicular to the screen plane.

13.5.2.4.3 Ortho Display Tab (Tomo3D)

Parameter	Description
Cut Lines	Sets the positions (pixel values) for the section lines using the **X/Y/Z** sliders or input fields. Alternatively you can also adjust the positions directly in the image area. To adjust the positions, move the mouse over a section line in the image. Hold down the left mouse button and move the mouse.
- Mid	Positions the relevant slider at the center of the view.
Line Width	Only visible if the **Show All** mode is activated. Enter the thickness of the section lines in pixels using the sliders or input fields. This results in a maximum intensity projection being displayed over the selected pixel width. You can also adjust the width directly in the image area. To adjust the width, move the mouse over a section line in the image until a small arrow is displayed. Hold down the left mouse button and move the mouse.
Cut Line Opacity	Only visible if the **Show All** mode is activated. Here you can enter the degree of opacity of the section lines from 0% (invisible) to 100% (completely opaque).
Views	Only visible if the **Show All** mode is activated. Sets the number and allocation of views in the **Image View**.
-	Displays four views.
-	Displays one view.
-	Displays two vertically separated views.
-	Displays two horizontally separated views.
-	Displays three vertically separated views.
-	Displays three horizontally separated views.

13.5.2.4.4 Appearance Tab (Tomo3D)

Here you can define the appearance of the 3D volume. On the tabs available on this tab, select the setting that you want to change (e.g. Transparency).

13.5.2.4.4.1 Transparency Tab

Parameter	Description
Channel selection	Here you can select the channel of a multichannel image for which you want to set the transparency.
Threshold	Sets the lower threshold value in percent of the gray levels displayed. With this setting you specify the gray value range for the relevant channel that you want to be included in the rendered image.
Ramp	Sets the extent of the transition from completely transparent to completely opaque (0-100 percent).
Maximum	Sets the level of opacity (0-100 percent).
Histogram	Displays the settings that you enter using the sliders schematically. The X axis represents the gray level values and the Y axis the opacity. You can also change the position of the curve using the mouse.
Reset	Resets all parameters to the original values.

13.5.2.4.4.2 Background Tab

Parameter	Description
Background Color	Sets the background color for the 3D view. To do this, click on the color field and select the desired color.
Reset	Resets the background color.

13.5.2.4.4.3 Light Tab

Parameter	Description
Brightness	Sets the brightness of the light source.
Reset	Resets the brightness.

13.5.2.4.5 3D Tab (Tomo3D)

Here you can specify which projection/rendering mode you want to use to display the 3D volume. There are three view modes available. To activate the desired view mode, click on the corresponding button.

Parameter	Description
Transparency	Activates the Transparency rendering mode.

Parameter	Description
Volume	Activates the Volume mode.
Maximum	Activates the Maximum intensity projection mode.

13.5.2.5 Analysis View

Only visible if an image analysis has been performed.

The **Analysis** view displays the following:

- the image from the analysis
- the table containing the analysis results
- a scatter chart or histogram

Info

- To highlight the row of the table containing the measured values of an object, click on a segmented object in the image or in the chart. To highlight multiple rows, press *Ctrl* and click on multiple object/ data points.
- To highlight the corresponding segmented object in the image, click on a row in the table or on the data point in the chart. To highlight multiple objects, press *Ctrl* and click on multiple rows/ data points.
- To highlight the measured value of an object in the scatter chart or in the histogram, click on one or more rows in the table. The corresponding data point in the chart turns red. To change the chart type, on the **Custom Chart** tab, click on the corresponding **Chart Type** button. To highlight multiple objects, press *Ctrl* and click on multiple rows/ objects.

You can also move your stage in the **Analysis** view if you click on the **Stage** button in the **Dimensions** tab and then on a position in the image. The stage is displayed as a red crosshair. This allows you to move your stage to particular points of interest which your analysis detected but would have been very hard to identify in the original image in the 2D view.

⚠ CAUTION

Risk of Crushing Fingers

The drive of a microscope stage with a motorized horizontal stage axis (stage drive) is strong enough to crush fingers or objects between the stage and nearby objects (e.g. a wall).

- Remove your fingers or any objects from the danger area before moving the stage drive.
- Release the joystick immediately to stop the movement.

See also

Charts and Tables of the Analysis View [393]
Custom Chart tab [857]

13.5.2.5.1 Analysis Tab

On the **Analysis** tab you can define how the measured objects are displayed in an image.

Parameter	Description
Show Objects	**Activated:** Displays the measured objects in the graphics plane.
Fill	**Activated:** Displays the objects in filled form. **Deactivated:** Displays only the contours of the objects.
Opacity	Here you can set the opacity with which the measured objects are displayed in the graphics plane.
Delete Measurement Data	Deletes all objects and measurement data from the image.
Show All Classes	**Activated:** Displays the objects of all classes. **Deactivated:** Displays the objects of the selected class.
Create Table(s)	Creates data tables
- <Classname>	Create data table for selected class.
- All classes (separately)	Create individual data tables for all classes.
- All class/classes (concatenated)	Create concatenated data tables for all class/classes.
Classes	In the Classes section you can select the class whose measurement features you want to be displayed in the measurement data table. For each class there are two entries: the first entry concerns all the objects belonging to the class (field features) and the second represents an individual object (object features).

13.5.2.5.2 Chart Export Tab

Here you can export the currently displayed custom chart as an image (with the file format PNG, TIF, BMP, or JPG).

Parameter	Description
Screen Resolution (96ppi)	Exports the chart with a resolution suitable for screens.
Printing Resolution (300ppi)	Exports the chart with a resolution suitable for printing.
– Width (inch)	Adjusts and displays the width.
– Height (inch)	Adjusts and displays the height.
– Maintain aspect ratio	**Activated:** If height or width is changed, the other parameter is changed automatically to maintain the aspect ratio. **Deactivated:** Height and/or width can be changed without an automatic adjustment of the other parameter. The aspect ratio is not maintained.

Parameter	Description
Transparent Background	**Activated:** The chart is exported with a transparent background. Possible file formats for this kind of export are PNG and TIF.
Export	Opens a dialog to select the folder and path for export.
Export Data	Available for histogram charts and the heatmap. Exports the data of the histogram or the heatmap as a *.csv file.

13.5.2.5.3 Sample Carrier Tab

On this tab you select the data which is shown in the multiple scenes chart of the **Analysis** view. Filled wells which contain objects (images) are displayed in yellow color, unfilled/ empty wells are transparent.

See also

Selecting data for the multiple scenes chart [▸ 396]

13.5.2.5.4 Table Export Tab

This tab allows you to export the currently opened table. The table is exported in csv format.

Parameter	Description
Export Table	Opens a explorer to select the location for export.

13.5.2.5.5 Custom Chart tab

You can display a histogram or visualize the relationship between two quantitative variables by scatter chart and adapt the zoom factor to optimize the display. Draw a rectangle into the histogram / scatter chart to zoom in. Double click into the histogram / scatter chart to zoom out.

Parameter	Description
Enable chart	**Activated:** Displays the chart in the **Analysis View**. **Deactivated:** Displays no chart in the **Analysis View**. Deactivates all controls described below to edit the chart.
Chart Type	
– Scatter Chart	Displays a 2D scatter chart in the **Analysis View**.
– Histogram Chart	Displays a histogram in the **Analysis View**.
X-Axis	Selects the feature that is displayed on the x-axis of the chart. The elements displayed in the drop down menu depend on the previously defined measurement features in the image analysis setting.
Y-Axis	Only available for the scatter chart. Selects the feature that is displayed on the y-axis of the chart. The elements displayed in the drop down menu depend on the previously defined measurement features in the image analysis setting.

Parameter	Description
Histogram Bin Count	Only available for the histogram chart. Creates the amount of bins to be displayed.
Adapt Zoom Factor	**Activated**: Automatically zooms the chart when selecting items from the data table or the image.
Multiple Scenes	Only available for multi-scene images. **Activated**: Displays a histogram/ scatter chart that contains the data points from all scenes. Deselect to go back to single-scene results.
Time Series	Only available for time series images. **Activated**: Displays the analysis results of the selected class in a time series chart.
Heatmap	Only available for experiments with multi well/ multi chamber plates. **Activated**: Displays a heatmap of the well plate.

See also

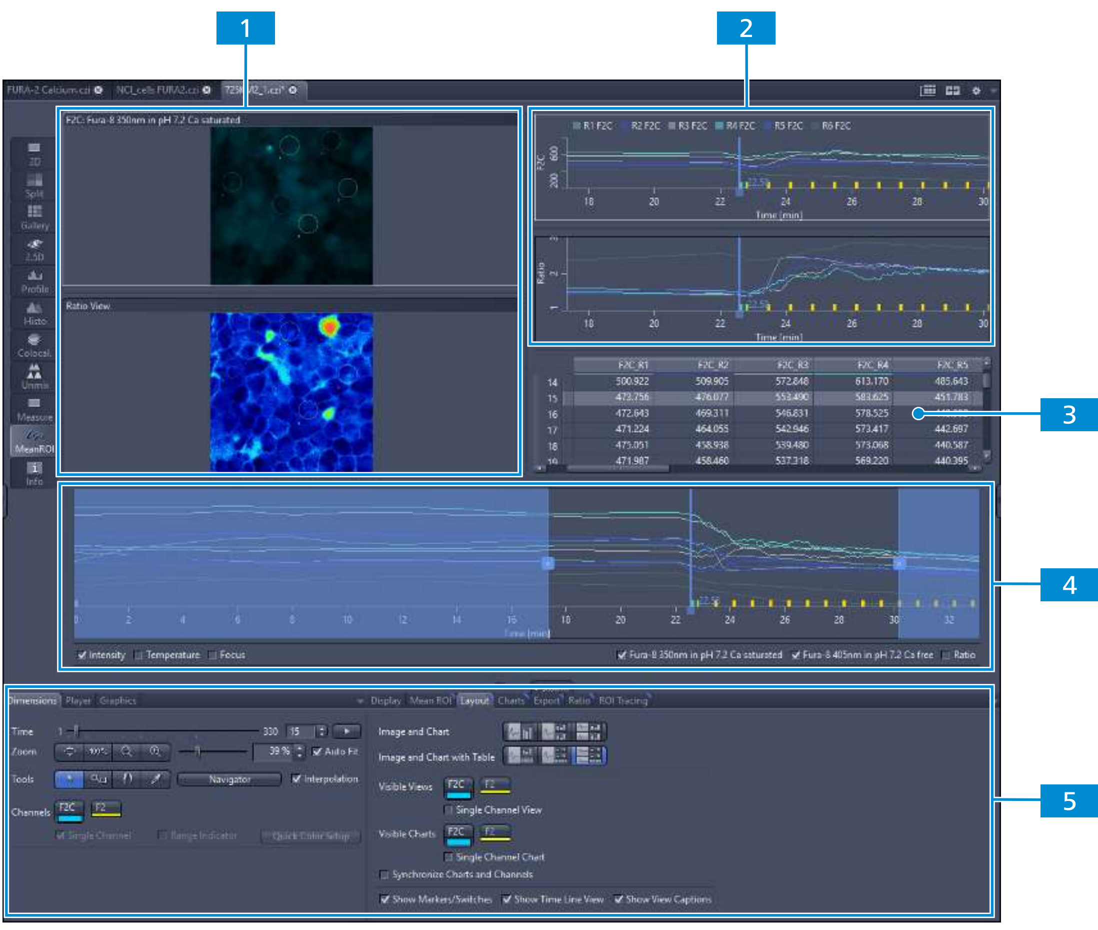
Analysis View [▸ 855]

13.5.2.6 Mean ROI View

In the **Mean ROI** view you can draw ROIs and measure their intensity profile after acquiring time series experiments. The intensity profiles are displayed as charts and can be exported to data tables.

Info

- The **Physiology (Dynamics)** module activates additional features to those of Mean ROI for the offline analysis of physiology experiments, e.g. the **Time Line View** panel, Ratio functions and ROI tracing.
- In this view the Image area is always to the left, charting area always to the right. Depending on which Region layout you have selected in **Layout** tab, the **Mean ROI** view can have a different appearance.



1 **Image area**
Here you see the images for each channel of the time series and the ratio image (if the ratio calculation is enabled). The display of images can be adapted in the **Layout** tab.

2 **Charting Area**
Here you see the charts for the values of all channels selected in the **Layout** tab as well as for the ratio calculation (if it is enabled on the **Ratio** tab). If a ROI is selected in the image area on the left, the corresponding plot line is highlighted (the plot line is thicker) in the charts.

Playhead (blue line)
Indicates the current frame of the time series visible in the image panel(s). The position of the playhead is synchronized with the displayed image frame number and vice versa. Press and hold the left mouse and drag the playhead in the desired direction. The current time point of the visible frame is displayed to right of the playhead line in the same time unit as the x-axis of the chart.

3 **Table**
This table displays the values for all channels and regions at the different time points, as well as the temperature, focus (if present in the image metadata) and information about markers. If you deactivate a channel in the **Visible Charts** section of the **Layout** tab, the corresponding columns are hidden in the table.
This table is synchronized with the image view and the charts. If you select a field in the table, the corresponding ROI is selected in the image view and the charts (playheads) are updated accordingly.

4 **Time Line View**
This view is only available with the **Physiology (Dynamics)** module and if it is activated in the **Layout** tab. The chart supports similar functions as the other charts in MeanROI. Here you can limit the time range with the zoom functionality. The actions in this chart are synchronized with the others in the MeanROI view. For more information, see *Time Line View panel [▶ 861]*.

5 **View Options**
Here you have your standard view options as well as specific options for MeanROI, for example for the **Layout** or the calculation of the **Ratio**.

Info

Hover over the plot with the mouse (crosshair). A tool tip appears with details of the intensity value at this position, ROI ID #, Channel, and time point (in currently set time unit of x-axis). Note these values (intensity and time) are interpolated. You can visualize the time points alone a plot by activation of the **Show Tick marks** function on the **Chart** tab.

See also

General View Options [▶ 887]

13.5.2.6.1 Time Line View panel

Only visible if you have licensed the **Physiology (Dynamics)** module.

This chart supports similar functions as detailed for charts in **Mean ROI**.

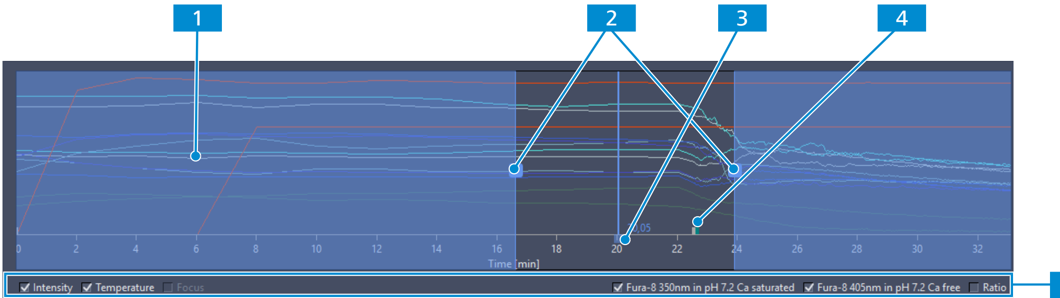


Fig. 73: Time Line View Panel

1 Intensity data traces from measurement ROIs.

2 Zoom control

The transparent area (blue) is the user definable zoom range. The zoom range translates into the display range of the x-axis of the other charts displayed above the time line view. To edit the zoom range hover use the controls at either end. Click and hold the left mouse to drag and resize the zoom area.

3 The center line (blue line) displays the playhead.

4 Markers with corresponding color code.

5 Chart data/intensity parameter selection check boxes

Depending on the available metadata different check boxes are available on the left side. Only two parameters can be displayed at a given time. The combination can be chosen as required.
When intensity parameters are displayed on the right side, it is possible to further select which of the available channels and/ or ratio should be displayed. Channel intensity and ratio intensity can be displayed in a mutually exclusive manner.

13.5.2.6.2 Mean ROI Tab

Parameter	Description
Background Correction	If you have activated the **Live Ratio Generation** in the **Online Ratio** tab, or the **Ratio Calculation** in the **Ratio** tab, the background correction is disabled here and only visible in the **Online Ratio**/ **Ratio** tab. Also note that the correction for ROI is only available if there are at least two ROI defined in the image! The following modes are available:
- None	No background correction is performed.
- Constant	Allows a user defined numeric value to be entered for both channels in the spin box.
- ROI	Allows to select the background ROI, the determined value will be channel specific.

Parameter	Description
Layout and Charts Default Settings	
- Define Default	Defines the current layout and chart setup as the default. The layout and charts can be changed in the **Layout** tab.
- Apply Default	Displays the default setup for layout and charts.
Restart Measurements	Only visible if the measurements calculation was canceled. Restarts the measurements calculation.

13.5.2.6.3 Layout Tab

Parameter	Description
MeanROI View Layouts	Adjusts how the images, charts, and the table will be displayed.
- Image and Chart	Selects one of three different layouts of how an image together with a chart will be displayed. If you click on one of the buttons the layout will be changed.
- Image and Chart with Table	Selects one of three different layouts of how an image and a chart together with a table will be displayed. If you click on one of the buttons the layout will be changed.
Visible Views	Selects the channels for which the image should be displayed in the image area.
- Single Channel View	**Activated:** Only one channel can be selected whose image is displayed. **Deactivated:** You can manually switch on/off channels whose images should (not) be displayed.
Visible Charts	Selects the channels for which the charts and table columns should be displayed in the chart area and the table.
- Single Channel View	**Activated:** Only one channel can be selected whose chart and information is displayed. **Deactivated:** You can manually switch on/off channels whose charts and information should (not) be displayed.
Synchronize Charts and Channels	**Activated:** Synchronizes the chart and channel settings.
Show Markers/ Switches	**Activated:** The temporal position of any switches and markers are always displayed on the charts both during acquisition or post-acquisition.
Show Time Line View	Only visible if you have licensed the **Physiology (Dynamics)** module. **Activated:** The Time Line View panel is displayed below the other image chart panels of the **Center Screen Area**. The Time Line View panel is designed to provide an overview of the experiment whilst allowing the user to examine the detail displayed in the other chart panels by means of an integrated zoom tool. The Time Line View can be hidden by unselecting the check box as required both during or after an experiment.

Parameter	Description
Show View Captions	**Activated:** Displays the channel name clearly with the image of each channel in the multichannel view layout. **Deactivated:** Hides the channel name of each image in the image view.

13.5.2.6.4 Charts Tab

Parameter	Description
All Chart Settings (X- / Y-Axis)	Note that a function is active when the button is highlighted in blue. The settings for **X-** and **Y-Axis** (only if **Show All** is activated) are the same, see description below. The Y- axis settings are always applied to the selected chart. The currently selected chart name (channel) is displayed above the Y-axis settings.
- Auto	The scaling of the respective axis is automatic, allowing for an optimal, and appropriate adjusting display of the all values.
- Fixed	The upper and lower limit of the axis can be defined using the min and max spin boxes.
X-Units	
- Auto	The units are selected automatically.
- Fixed	You can select the desired unit for the x-axis from the dropdown list.
Show Tick Marks	Shows tick marks in the chart. If activated, you can set the **Form** and **Size** of the tick marks. Currently, the tick marks have to be set per chart.
Show Legend	Shows the chart legend.
Show Axis Captions	Shows captions of the axis.

13.5.2.6.5 Export Tab

Parameter	Description
Data Table	
- As New Document	Opens the measurement data table in a new document tab. The table displays all measurement values and area for all ROIs in each channel. If event markers are present these are also listed here at the appropriate time points. For a description of the measured parameters, see *Basics of Calculation of Intensity and Ratio Values [463]*.
- Save as *.csv	Opens the **Save As** dialog and allows the measurement data to be exported as a comma separated value (***.csv**) file. The following values are exported for each ROI and channel: Intensity, area and if present event markers. The exported values are the raw data without the subtraction of any background correction.

Parameter	Description
Ratio image	
- As New Document	Opens the ratio image in a separate new document as a *.czi file (current Z only).
- Save as	Opens the **Save As** dialogue to save the ratio image directly to a ***.czi** file.

13.5.2.6.6 Ratio Tab

This view option is almost identical to the **Online Ratio** tab in the **MeanROI Setup**, see *Online Ratio Tab [▸ 864]*. In fact when an experiment is finished the exact same values used for the display of the online ratio are transferred to the offline ratio tool of the **MeanROI** view and are stored with your image for later reference.
For offline ratio assessment the settings can be changed and are applied to the ratio image and the measured values. For large data sets (high resolution or many time points), a smooth playback of the ratio image can be achieved by clicking on the **Cache ratio image** button. This stores the ratio images temporarily to the computers memory for very fast playback or adjustments of the time slider. The following descriptions describe the differences on the Ratio tab:

Parameter	Description
Ft_0= Average intensity of frame	Only available if the **Single wavelength** method is selected. Defines the frames of the time series image from which the reference value Ft $_0$ should be calculated. The numbers in the input fields refer to the frame number of the current experiment in the **MeanROI** view. The desired frame numbers can be entered with the buttons or directly by typing a number into the field. Typically, your experiment should include a baseline of 5-10 images before and/ or in between a simulation/ activation.
– Update	Applies the changes of the frame made with the input fields.
Cache ratio image	Caches all the ratio images of the current time series with the given ratio calculation parameters. This is done to avoid image flickering when moving through the time series with the slider quickly.

13.5.2.6.7 Online Ratio Tab

Parameter	Description
Calculation dropdown list	Selects the ratiometric method you want to use. **Single** and **Dual wavelength** dyes are supported with an additional three formulas for further adapted (online or offline) image ratio calculations. The ratio set-up will change in accordance with your selection.
- Single Wavelength Method	Select the channel in the dropdown menu. The Ft_0 value is the averaged fluorescence from the specified number of image frames. The number of frames to average is defined in the spin box of the reference image set-up (see 10). The spin box at the far left is a multiplication factor.

Parameter	Description
- Dual Wave-length Method	Select the channels in the dropdown list required to calculate the ratio values/image e.g. for Fura-2, a dual excitation dye, the numerator is the 340 nm image the denominator the 380 nm image. For dual emission dyes the function is identical. The spin box at the far right is a multiplication factor.
- Image Ratio Type 2	The formula calculates the normalized ratio of the difference between two weighted channel intensities.
- Image Ratio Type 3	The formula calculates the ratio between the weighted difference and weighted sum of two channel intensities
- Image Ratio Type 4	The formula calculates the ratio between the intensity difference of two channels in relation to the intensity of one channel.
Background Correction	A background correction can be performed on a channel by channel basis. The selection of a background correction method modifies the ratio set-up formula accordingly. Note that the correction by ROI is only available if there are at least two ROI defined in the image! The following modes are available:
- None	No background correction is performed.
- Constant	Allows a user defined numeric value to be entered for each channel in the appropriate spin box.
- ROI	Allows to select the background ROI defined in the **Mean ROI** view/ setup. Note that for dual wavelength protocols the same ROI is used in each case, but its channel specific values are applied for the correction. For single wavelength ratio, no ROI background correction is available.
Ratio Clipping	**Activated:** Sets the factor for ratio clipping.
Color	Select the color (LUT) used to display the ratio image. Per default the Rainbow LUT is used as it allows intensity changes to be followed easily.
Enable Threshold	**Activated:** Allows the threshold values to be set for the ratio calculation.
- Channel / Threshold	A threshold value can be applied in the form of a constant integer value for each channel individually. Thresholds help to reduce noise anomalies that are cause by pixel to pixel variations in areas between cells or near cell boarders during the ratio calculation. Enter the desired threshold value for each channel into the spin boxes provided. For more detailed information on how ZEN handles thresholds, see *Basics of Calculation of Intensity and Ratio Values [▸ 463]*.

13.5.2.6.8 ROI Tracing Tab

This tab is only available if you have licensed the **Physiology (Dynamics)** module.
ROI tracing allows you to adjust the position of your ROI as necessary to accommodate the lateral movement of an object in a image of which the mean intensity is to be measured. This is done by defining a series of one or more so called key frames for individual ROIs. In this manner, complex object movements can be corrected.

Parameter	Description
Enable ROI Tracing	Enables the functionality for ROI tracing.
Selected ROI	Displays the number and shape of the currently selected ROI.
Key Frame Edit Mode	Only available if you have selected a ROI.
– **All Key Frames**	Manipulates the ROI for all time points/key frames.
– **Single Key Frame**	Manipulates the ROI for the currently selected time point and creates a key frame. Note that a single key frame adjustment can only be performed when the frame number (time point) is set to 2 or higher.
Interpolation	Only available if you have selected a ROI. Selects an interpolation method for the ROI changes between the time points.
– Constant	Does not interpolate the ROI position between the key frames, i.e. the ROI is pnly present at the set key frames.
– Linear	Determines the ROI position at the time points between key frames based on linear interpolation.
– Spline	Determines the ROI position at the time points between key frames based on spline interpolation.
Key frame list	Displays the key frames of the ROI and the changes compared to the previous key frame.
– **Add**	Adds the current position as key frame.
– **Delete**	Deletes the currently selected key frame.
Show	
– Trajectories	**Activated:** Displays the trajectories between the key frames in the image.
– Ticks	**Activated:** Displays ticks for the (center) position the ROI for each time point in the image. These ticks are only visible if **Linear** or **Spline** is selected as interpolation method.
– Ghosted key frames	**Activated:** Displays the shape of the ROI at each key frame. **Deactivated:** Displays the shape of the ROI only at the currently selected time point.

Parameter		Description
–	All ghosted	Only available if **Ghosted key frames** is activated. **Activated:** Displays the shape of the ROI for all time points. This effect is only visible if **Linear** or **Spline** is selected as interpolation method.

See also

Adjusting ROIs for Time Points [▶ 459]

13.5.2.7 Unmix View

This view is only visible for multi-channel or lambda stack images. It is used for:

- display the spectra corresponding to defined ROIs (mean ROI intensity over Lambda),
- show the intensity values in table form, copy the table to clipboard or save the table as a text file and
- generate linear unmixed multi-channel images.

In this view you will see 2 areas as default. The intensity-over-lambda diagram **(1)** to the left and the image display **(2)** to the right. The specific view options **(3)** below the image area are described in the following topics.

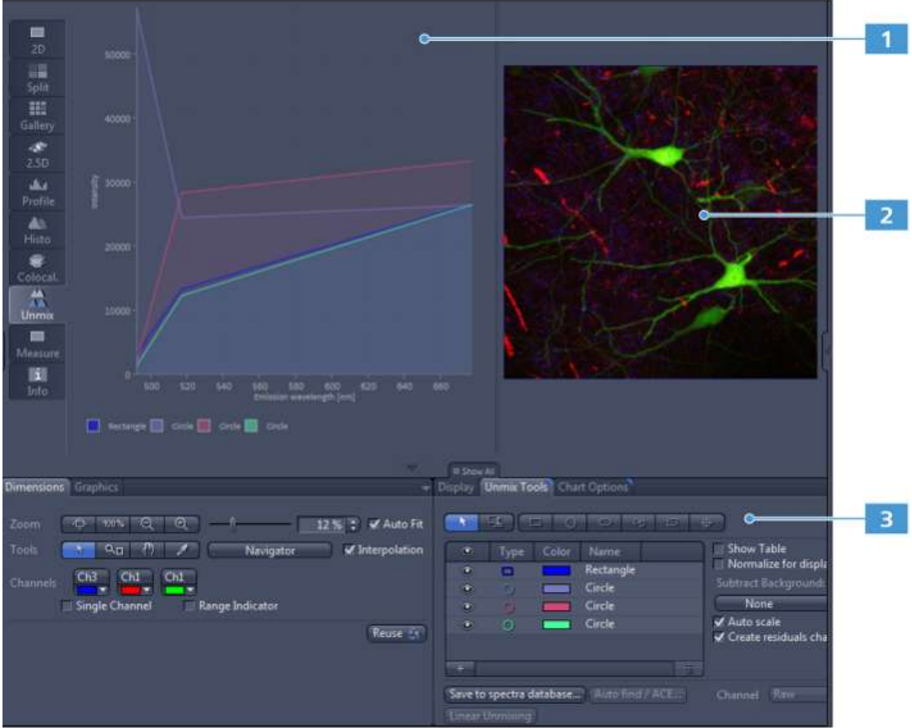


Fig. 74: Unmix View

13.5.2.7.1 Unmix Tools Tab

Here you can select various tools and use these to draw graphic elements into your images, similar to the tool bar on the **Graphics** tab. You can also obtain an overview of the graphic elements that you are using in your image.

The following list will describe the specific parameters for this tab:

Parameter	Description
Toolbar	Using the tools you can draw in certain regions of interest which are then displayed in the intensity-over-lambda diagram and will be used for linear unmixing.
- **Select**	Use this to select the graphic elements in the image area. If you are currently in another mode, you can switch back to the selection mode using this button.
- **Clone**	Use this to create an identical copy of the last graphic element drawn in by simply clicking anywhere into the image area. To exit this mode, either switch back to the selection mode or press the *ESC* key.
- **Draw Rectangle**	Draws a rectangular Region of Interest (ROI) into the image.
- **Draw Circle**	Use this to draw in a circle. This element by default also shows the mean gray level of the image region.
- **Draw Ellipse**	With this tool you can draw a elliptical region.
- **Draw Spline Contour**	Use this to draw in a freely selectable contour. You can either define the corner points by a series of clicks or you can trace a contour by keeping the left mouse key pressed. Close this contour by right-clicking. Corners are always rounded with this tool. This element by default also shows the mean gray level of the image region.
- **Draw Polygon**	With this tool you can draw a polygonal region.
- **Draw Cross**	With this tool you can draw a cross marker into the image, where the pixel at the center of the cross is taken for the unmixing measurement. **Note:** The 10x10 pixel rectangle around the cross is only a visual aid to locate the marker, the unmixing is only taking the one pixel at the center into account and not this rectangular bounding box.
List of Spectral Data	The list gives you an overview of the spectral data in the image, which will be used for linear unmixing. The names indicates the origin, e.g. if manually or automatically picked by **ACE** (see below) or loaded from the spectra database. To load a spectrum from the spectral database, press the Add **+** button for a new row. Click into the Name column and select the according name for the needed spectrum.
Save to spectra database	If you click on this button you can save the selected entry to the spectra database.
Auto find /ACE...	ACE stands for Automatic Component Extraction. If you click on this button the software automatically searches for regions with distinct spectral signatures and tries to find the defined number of spectra.

Parameter	Description
Linear Unmixing	Performs the linear unmixing processing of the image with the selected spectra. Note: The channels of the Lambda stack which are de-selected in the **Dimensions** tab are not included in the calculation.
Show Table	**Activated**: Displays a table of intensity values over Lambda below the default image area.
Normalize for display	**Activated:** Normalizes the graphs of the spectra to 1.
Subtract background	Here you can select the list entry of a marked spectrum that should be subtracted before linear unmixing.
Auto scale	**Activated:** Automatically balances the intensity of unmixed channels to equal levels.
Create residuals channel	**Activated:** Generates an additional channel in which the intensity value represent the difference between the acquired spectral data and the fitted linear combination of the reference spectra for each pixel. In essence, the residual value is the biggest remaining "residual" from the least square fit routine. The residuals are a general measure for how good the fit of the algorithm has performed.
Channel	
- Raw	The raw data acquired during a lambda stack is used as channels and for spectral display.
- Spectral	The intensity data of the lambda stack is calculated into channels for each detector (Channel 1 and Channel 2).

13.5.2.7.2 Chart Options Tab

This tab provides several parameters to change the appearance and contents of the spectral graph. For the beginning we recommend to use the default settings here.

13.5.2.8 Lambda View

In this view you can display images that are acquired in Lambda mode, see Lambda Mode. the resulting images are called Lambda stacks. For that type of image the **2D** View is not available.

Instead the Lambda View displays a Lambda Stack in a wavelength-coded color view as default. A color palette, mimicking the emission wavelength of the channel, is automatically assigned to the individual lambda images which are then displayed in a merge-type display.

On **Display** tab, the channel-specific settings of brightness, contrast and gamma can be handled as described for channels in the **2D** View.

In order to use other views (e.g. Split or Gallery view) or to view lambda stack data sets in ZEN black, convert the data set using the **Convert to Lambda** image processing function.

The general view options on **Dimension** tab are adapted to the Lambda View with the following changes:

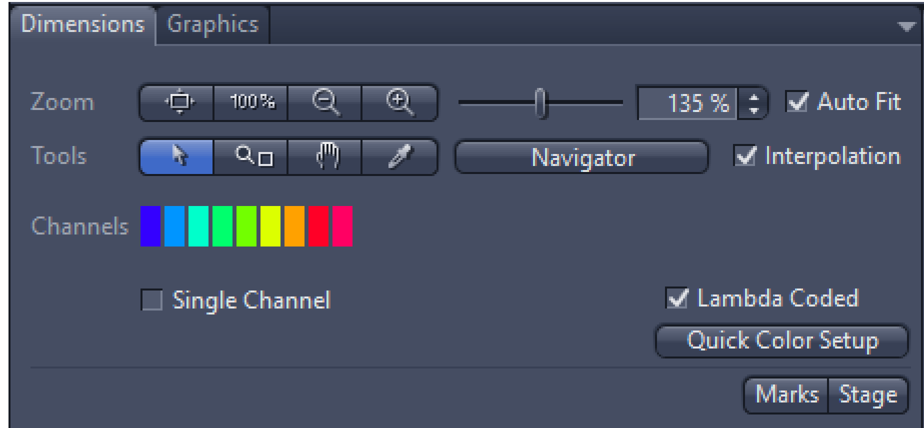


Fig. 75: Dimensions tab changes for Lambda view

Parameter	Description
Channels	Displays the single channels of a Lambda stack image as a colored button. You can handle the channels like in the 2D View. E.g. if you click on a channel button you can show or hide the channel in the image area.
Lambda Coded	**Activated:** All channels are displayed as a merged image. Each channel is assigned to a channel color that represents the recorded emission wavelength in the lambda stack. **Deactivated:** Only one channel of the Lambda Stack is displayed without pseudo coloring. Additionally the **Single Channel** checkbox is activated and cannot be changed. To display a different channel of the Lambda Stack, click on the according channel. This will display the chosen channel and deactivate the previously displayed channel.

13.5.2.9 Colocalization View

Only visible for multichannel fluorescence images.

In the **Colocal.** (Colocalization) view, you can analyze the extent of colocalization quantitatively in two fluorescence channels. The view consists of two main areas: the **X/Y scatter plot** on the left and the actual image (2 channels are displayed) in the right image area. Using the **Coloc. Tools** specific view control, you can also display the **Colocalization table** in the lower image area. To do this activate the **Table** checkbox in the **Extract** section.

Info

The channels that you are comparing with one another are displayed in the image area in the form of a color overlay. The channel color of the image is used here. If the images have more than 2 channels, you can add additional channels on the **Dimensions** tab. This temporary selection is deactivated, however, when you select the channels to be compared on the **Coloc. Tools** tab.

See also

General View Options [▸ 887]

13.5.2.9.1 X/Y Scatter Plot

The pixel intensities of two channels are plotted against one another in the diagram and each pixel pair with the same X/Y image coordinates is displayed as a point. The frequency with which pixels of a certain brightness occur is visualized by means of a color palette that is displayed at the bottom of the diagram. The relative value range lies between 0-255.

The vertical and horizontal axes show the gray value range that applies for the relevant channel.

The diagram is overlaid with two lines that subdivide it into 4 quadrants, numbered from 1-4. Using the mouse you can position the lines freely and therefore adjust the threshold values to the data.

The quadrants have the following meanings:

- 1: Non-colocalizing pixels from channel 1
- 2: Non-colocalizing pixels from channel 2
- 3: Colocalizing pixels
- 4: Background

13.5.2.9.2 Coloc. Tools Tab

Here you find all control elements you need to perform a colocalization analysis.

13.5.2.9.2.1 Tool Bar section

Only visible if the **Show All** mode is activated.

Use the tools to draw regions into the image in which you want the analysis to be performed. A description of the tools can be found on the *Graphics [894]* tab.

Once a region has been drawn in, it is automatically treated as an active region. The scatter plot shows the pixel value frequencies for this region.

The **Colocalization table** displays the data for the entire image and for the selected region. To select several regions, hold down the **Ctrl** key and click on the desired regions.

Apart from drawing regions into the image, you can also draw them into the **X/Y scatter plot**. If you have used the function in the *Regions section [873]*, only those pixels that are framed by a region in the scatter plot are taken into consideration. This means that you can correlate interesting point clouds quickly with the corresponding pixels in the image.

If you have drawn regions into the scatter plot, the ROI (region of interest) button will also appear in the tool bar. As long as this button is activated (highlighted in blue), you can select, move and change the regions in the scatter plot using the **Selection** tool. If you want to change the quadrant lines again, you will need to deselect the ROI button beforehand.

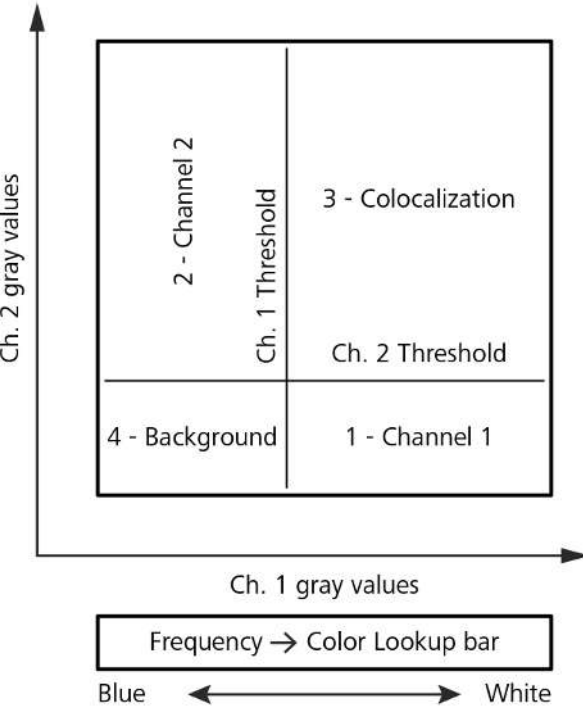
13.5.2.9.2.2 Channels and scatter plot control section

Channels dropdown lists

Here you can select which channels of a multichannel image are compared with one another. Select a channel for both the horizontal and vertical diagram axis from the **Channels** dropdown list. The first and second channel are always selected by default. As soon as you have made a selection, all other channels are automatically removed from the image display. You can, however, add other channels temporarily on the **Dimensions** tab.

Threshold sliders

Using the two Threshold sliders and the two spin boxes/input fields, you can set the threshold value (in gray levels) for both channels.



Range dropdown lists

Only visible if the **Show All** mode is activated.

Here you can define the gray value range that the scatter plot axes will display. **Auto** is selected here by default, which means that the range is automatically set to the brightest pixel in the image. You can, however, enter a fixed gray value range between 256 (8 bits) and 65535 (16 bits). If the image is a time lapse or Z-stack image, **Auto** has been selected, you can select the dimension for which you want the gray value range to be automatically determined from another dropdown list. In this way you can easily determine a valid diagram setting for an entire time series, for example, without having to analyze each individual time point.

Dimension selection dropdown list

This dropdown list is only visible if at least one of the **Range** dropdown lists is set to **Auto** and if the image is a z-stack or a time series. Defines whether the axis of the scatter plot are based on the values of the current image plane (**2D**), or on the entire z-stack (**All Z**)/time series (**All T**).

Costes button

Calculates the optimal threshold value according to Costes et al.

13.5.2.9.2.3 Regions section

Channels buttons

Here you can mask pixels in the image according to which one of the four quadrants they belong to. The numbers on the buttons correspond to the numbering of the quadrants in the **X/Y scatter plot**. The color selection window is accessed by clicking on the **color field**. Using the **Opacity** slider you can determine the degree of transparency of the masking.

Cut Mask button

Only active if a quadrant has been masked.

Creates a new image exclusively containing the masked pixels.

13.5.2.9.2.4 Extract section

Scatter Plot button

Creates a new image document from the X/Y scatter plot. In the case of time series or Z-stacks the dimensions are also created automatically.

The following functions are only visible if the **Show All** mode is activated:

Table button

Creates a new table document. The document contains all measurement data from the colocalization analysis. All dimensions, such as T and Z, are also taken into account. This table can be saved as a *.csv document for further processing in other programs.

Table checkbox

Activated: Displays the colocalization table in the image area.

13.5.2.9.3 Colocalization table

Only visible if the **Table** checkbox is activated on the **Coloc. Tools** tab.

For each quadrant of the scatter plot there is a correspondingly labeled row in the table. The **Global** row contains the values for the entire image. The table contains columns for the following measured values:

13.5.2.9.3.1 Region

Once a region has been selected it has a number assigned to it. This number appears in the image and in the table.

13.5.2.9.3.2 Quadrant

Indicates the measured values for the four quadrants of the scatter plot.

13.5.2.9.3.3 Pixel Number

Shows the total number of pixels of each quadrant. The sum of all pixels in this column for all 4 quadrants corresponds to the product of the height x width of the original image.

13.5.2.9.3.4 Area (µm²)

Area = number of pixels x scaling factor for X/Y
If there is no scaling for the original image, the following applies: 1 pixel = 1 µm.

13.5.2.9.3.5 Relative Area (%)

Relative area = area of quadrant/total area

13.5.2.9.3.6 Pearson's Correlation Coefficient

Provides information on the intensity distribution within the colocalization region.
Value range: -1 to 1.

1: All pixels are on a straight line in the scatter plot from bottom left to top right (if, for example, you have used the same channel twice for the colocalization, you will find the value 1 in this column).

0: The pixels in the scatter plot are distributed in a cloud without a preferred direction.

-1: The pixels do not overlap. The scatter plot stretches from top left to bottom right. This situation can be described as negative colocalization and means "exclusion".

The calculation formula is as follows:

$$\frac{\sum((GreyCh1_i - MeanCh1) \times (GreyCh2_i - MeanCh2))}{\sqrt{\sum(GreyCh1_i - MeanCh1)^2 \times \sum(GreyCh2_i - MeanCh2)^2}}$$

GV: Gray Value; AV: Average Gray Value; C: Channel

13.5.2.9.3.7 Manders' Correlation Coefficient

Insensitive to differences in the signal intensity between the two channels and bleaching.

Value range: 0 to 1

The calculation formula is as follows:

$$\frac{\sum GreyCh1_i \times GreyCh2_i}{\sqrt{\sum GreyCh1_i^{\,2} \times \sum GreyCh2_i^{\,2}}}$$

Fig. 76: C: Channel

13.5.2.9.3.8 Coloc. Coefficient 1

This coefficient indicates the relative number of colocalized pixels in channel 1 in relation to the total number of pixels above the threshold value:

$$\frac{\sum PixelsCh_{1,colocalized}}{\sum PixelsCh_{1,total}}$$

The values range between 0 and 1, with 0 indicating no colocalization and 1 indicating full colocalization.

Numerator = Number of pixels in quadrant 3

Denominator = Number of pixels in quadrant 3 + number of pixels in quadrant 1

13.5.2.9.3.9 Coloc. Coefficient 2

This coefficient indicates the relative number of colocalized pixels in channel 2 in relation to the total number of pixels above the threshold value:

$$\frac{\sum PixelsCh_{2,colocalized}}{\sum PixelsCh_{2,total}}$$

The values range between 0 and 1, with 0 indicating no colocalization and 1 indicating full colocalization.

Numerator = Number of pixels in quadrant 3

Denominator = Number of pixels in quadrant 3 + number of pixels in quadrant 2

13.5.2.9.3.10 CC (weighted) 1

Weighted correlation coefficient channel 1. Calculated like the simple colocalization coefficient, but using the sum of the gray value intensity rather than the number of pixels.

$$\frac{\sum SumGreyCh_{1,colocalized}}{\sum SumGreyCh_{1,total}}$$

The values range between 0 and 1, with 0 indicating no colocalization and 1 indicating full colocalization.

Numerator = Sum of intensity of all pixels in quadrant 3

Denominator = Sum of intensity of all pixels above the threshold value

13.5.2.9.3.11 CC (weighted) 2

Weighted correlation coefficient channel 2. Calculated like the simple colocalization coefficient, but using the sum of the gray value intensity rather than the number of pixels.

$$\frac{\sum SumGreyCh_{2,colocalized}}{\sum SumGreyCh_{2,total}}$$

The values range between 0 and 1, with 0 indicating no colocalization and 1 indicating full colocalization.

Numerator = Sum of intensity of all pixels in quadrant 3

Denominator = Sum of intensity of all pixels above the threshold value

13.5.2.9.3.12 Average Intensity 1

The sum of all gray values from channel 1, divided by the total number of pixels in this channel:

$$\frac{\sum GreyCh1_i}{AreaCh_1}$$

13.5.2.9.3.13 Average Intensity 2

The sum of all gray values from channel 2, divided by the total number of pixels in this channel:

$$\frac{\sum GreyCh2_i}{AreaCh_2}$$

13.5.2.9.3.14 Standard Deviation 1

Displays the standard deviation of the gray values in channel 1:

$$\sqrt{\frac{\sum(GreyCh1_i - MeanIntensityCh_1)^2}{AreaCh_1 - 1}}$$

13.5.2.9.3.15 Standard Deviation 2

Displays the standard deviation of the gray values in channel 2:

$$\sqrt{\frac{\sum(GreyCh2_i - MeanIntensityCh_2)^2}{AreaCh_2 - 1}}$$

13.5.2.9.3.16 Z Index

Displays the Z index for Z-stack images.

13.5.2.9.3.17 T Index

Displays the time index for time lapse images.

13.5.2.9.3.18 Relative Time

Displays the time of acquisition for all dimensions of a multidimensional image, beginning at 0h:00min:00sec:00msec.

13.5.2.9.3.19 Relative Focus

Displays the relative focus position at which an image has been acquired.

13.5.2.10 FRAP View

The FRAP view (FRAP = Fluorescence Recovery after Photobleaching) is only visible for a time series data set which includes a minimum of one bleach event. It permits interactive analysis of bleaching experiments, including:

- Fit Formula
  Fitting of FRAP data to a mono exponential or double exponential model for intensity
- Fit Range
  Selection of data points for the fitting
- Photofading Factor
  Determining the fading factor from a reference region (Ref.) from the present experiment or a control experiment and reusing it for subsequent experiments

- Defining Background and Reference Regions for the analysis (fitting) of FRAP data

Note: When acquiring Airyscan SR or Airyscan MPLX data, FRAP View only works as expected with the complete time series date processed

The Image Display in the FRAP View shows 4 panels:

- the intensity-over-time diagram with the fitted curve per channel **(1)**
- the image display with the ROI graphics **(2)**
- the table with the fit parameters for each channel and analysis ROI Group (one or more ROIs can be grouped for analysis, up to three groups can be analyzed) **(3)**
- an optionally viewed table of the average intensity values of each ROI Group (corrected for Background and Reference) for each time point and channel

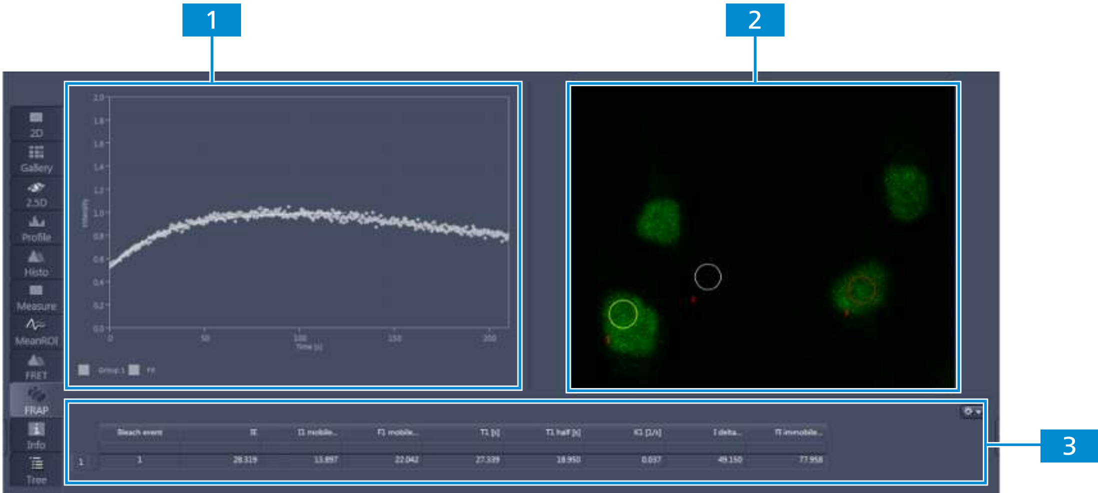


Fig. 77: Image display of a time series with bleach event in the FRAP View Tab

13.5.2.10.1 FRAP View Options

The additional view-specific FRAP View Options are available in the **FRAP** View Options Tab.

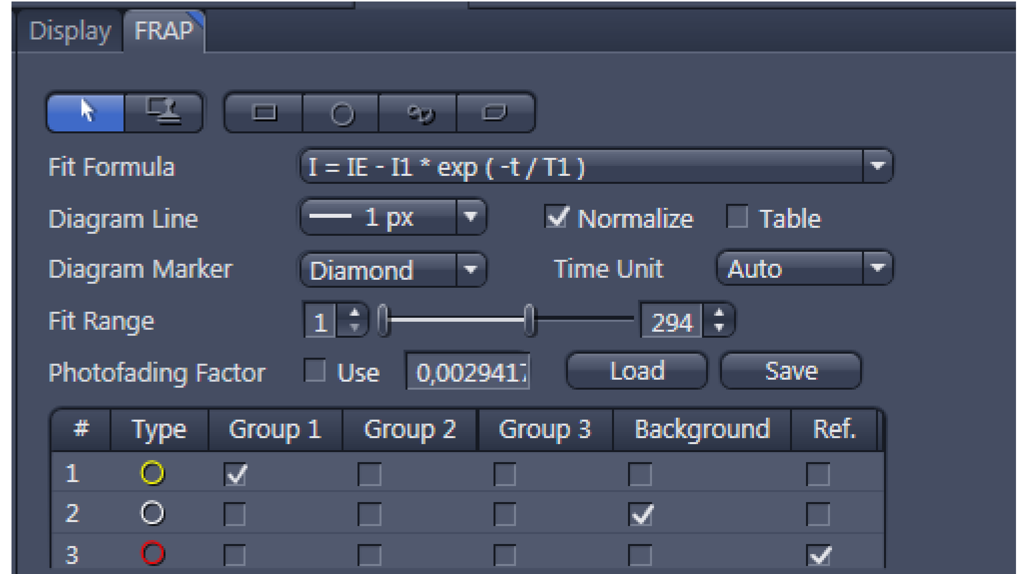


Fig. 78: FRAP View Options Tab

Any changes performed with this view options tab are effective immediately.

The settings of the Dimensions, Display, Player and Graphics View Options tabs apply for FRAP View, too.

Available tools in the **FRAP** View Options Tab are:

Parameter	Description
Regions of Interest (ROIs)	The **FRAP** View Options Tab includes drawing tools that work identically to the Graphics View Options Tab. ROIs from both control blocks can be combined.
Fit Formula	Drop down menu to select the mathematical model (mono or double exponential model) for data fitting
Diagram Line	Drop down menu to format the line thickness of the fit curve(S) in the diagram
Diagram Marker	Drop down menu to format the time point markers of the fit curve(s) in the fit diagram
Normalize	Checkbox to choose between absolute or normalized fit data. This updates the table of the intensity values and the fit diagram
Table	Checkbox to activate/deactivate the display of the intensity value (for each channel) per time point corrected for background and reference region if applied
Time Unit	Drop down menu to format the time units of the fit diagram, the fit data table and the intensity table
Fit Range	Edit boxes and slider to define the data range for the fit algorithm
Photofading Factor	Checkbox to Use or not use the photofading factor for the data fitting algorithm; Edit box to type in a photofading factor; Load and Save function buttons to save and retrieve a factor (.xml file). The photofading factor is either calculated from the current reference region(s) or it is loaded from a previous experiment. The edit box shows the currently (applied) value. The photofading factor = reference(t)/reference(t=0) and then fitted by: intensity(t) = exp(-kappa * t). The fitted value of kappa is displayed in the edit box.
Background Region	Check box in the list of ROIs: Mark the region of interest which represents the mean background intensity to be used for data correction. The mean intensity value of the background region is subtracted from the data prior to fitting.
Reference Region	Check box in the list of ROIs: Define and select a ROI which represents the fluorescence intensity of a reference area that has not been bleached and has not been affected by the leach event. The mean intensity within that region is used to correct the data at each time point for any bleaching artifact that occurred during the imaging process. For this the data are divided by [reference(t)/reference(t=0)]
Combine Regions Group 1, 2, 3	Check boxes in the list of ROIs which allow to choose more than one ROI for analysis and to group them according to the experimental set up. Mean Intensity values for the regions combined are used as data input.

13.5.2.10.2 Fit Model

The Fit Model applied provides the following data/parameter:

- The final signal intensity in the analyzed ROIs following recovery **IE** (of the fitted curve)
- The amplitude of the fitted curve (which equals the mobile fraction) **I1** mobile fraction
- The proportion of the mobile fraction: **F1 mobile fraction (%)**
- The fitted parameter **T1(s)**
- The half time of recovery **T1 half (s)**
- The rate constant for the exchange of molecules between the bleached region and the surrounding area **K1 (1/s)**
- The part of the immobile fraction of the protein **I delta immobile fraction**
- The proportion of the immobile fraction: **F1 immobile fraction (%)**

A double exponential displays the mean of the fitted values for the two different mobile fractions as the fit curve. The following (additional) parameters are provided

- The amplitude of the two curves, displayed as one (which corresponds to each part of the mobile fractions) **I1** and **I2**.
- The fitted parameters **T1 (s)** and **T2 (s)** for each mobile fraction.
- The rate constant for the exchange of molecules between the bleached region and the surrounding area **K1 (1/S)** and **K2 (1/s)** for each mobile fraction.
- The half time of recovery for each fraction **T1 half (s)** and **T2 half (s)**

The table displays the result of the data fit. The result can be copied to the clipboard (right mouse click) and directly pasted into excel or saved as text.

The calculation of the parameters is based on the bleached ROI(s) unless other ROIs or selected or the ROIs are moved.

The analysis is then part of the image and the type of analysis is displayed again when opening the FRAP view for the image.

13.5.2.11 FRET View

13.5.2.11.1 Introduction

Förster Resonance Energy Transfer (FRET) is a mechanism which describes an energy transfer between two chromophores, typically fluorescent proteins. Upon excitation with light of suitable wavelength a donor chromophore, then in its electronic excited state, can transfer energy to an acceptor chromophore. The efficiency of this energy transfer is dependent on the distance between the two molecules. This makes **FRET** an indicator for even very small changes in the distance between two molecules.

When using confocal or super resolution techniques **FRET** is typically used to determine whether two fluorophores are within a certain distance of each other and whether this distance changes due to external or internal influences.

See also

Acquisiton Prerequisites [880]
Sensitized Emission [880]
Acceptor Photobleaching [884]

13.5.2.11.2 Acquisiton Prerequisites

There are two ways to access **FRET** measurements:

- **Sensitized Emission**
- **Acceptor Photobleaching**

Different acquisition set ups are necessary for each approach to enable the **FRET** View.

Analysis along **Sensitized Emission** requires a multichannel (multidimensional) data set (minimum 3 channels). The acquisition settings are determined by used fluorophores. Refer to scientific papers for more information on sample preparation and necessary controls for sensitized emission experiments.

Analysis along **Acceptor Photobleaching** requires a time series with a bleach event. The acquisition settings are determined by used fluorophores. Refer to scientific papers for more information on sample preparation for acceptor photobleaching.

Depending on the data set **FRET** view provides different parameter sets for image analysis.

To access **FRET** View, the data set needs to be loaded or be completely acquired. Online calculation of **FRET** data is not supported.

See also

Sensitized Emission [880]
Acceptor Photobleaching [884]

13.5.2.11.3 Sensitized Emission

13.5.2.11.3.1 FRET tab

Parameter	Description
Graphics tools	Use graphics tools to define the region(s) to be analyzed including the (optional) region which defines background signal.
Method	Select the analysis method. ZEN provides three different methods for data analysis. Depending on the method selected the display of the **FRET** image changes. The data table displays the results of all three methods. For more information, see Analysis methods for Sensitized Emission.
Create Image	The displayed **FRET** image is displayed as new image document.
ROI table	Checkboxes assign the defined regions as region to be analyzed or background region.

13.5.2.11.3.2 Parameter tab

Determine the Donor coefficient values by using a sample with signal of the donor fluorophore only using the same imaging settings which are later used for imaging the FRET sample. Define one or more regions in the image which are enabled as objects to be analyzed in the **FRET** tab. The Donor coefficient values are taken from this region by clicking the **Analyze Donor** button.

Determine the Acceptor coefficient values by using a sample with signal of the acceptor fluorophore only using the same imaging settings which are later used for imaging the FRET sample. Define one or more regions in the image which are enabled as objects to be analyzed in the **FRET** tab. The Acceptor coefficient values are taken from this region by clicking the **Analyze Acceptor** button.

Parameter	Description
Donor Ch	Define the channel which contains the **Donor** signal.
Acceptor Ch	Define the channel which contains the **Acceptor** signal.
FRET Ch	Define the channel which contains the **FRET** signal.
Analyze	Select which signal has to be analyzed from the present image.
Donor coef. Fd/Dd	Shows the value of **Donor** signal detected in the **FRET** channel/Donor signal detected in the Donor channel from the analyzed present image.
Donor coef. Ad/Fd	Shows the value of **Donor** Signal detected in the **Acceptor** channel/ **Donor** signal detected in the **FRET** channel.
Acceptor coef. Fa/ Aa	Shows the value of **Acceptor** signal detected in the **FRET** channel / **Acceptor** signal detected in the **Acceptor** channel.
Acceptor coef. Da/ Aa	Shows the value of **Acceptor** signal detected in the **Donor** channel / **Acceptor** signal detected in the **Acceptor** channel.
Acceptor coef. Da/ Fa	Shows the value of **Acceptor** signal detected in the **Donor** channel/ **Acceptor** signal detected in the **FRET** channel.
G	Allows to set Gordon Factor which depends on the fluorophores used and which can be part of the Gordon and Xia analysis. Please refer to the original publications for further details (Gordon et al., Biophys J 74, 2702 (1998), Xia and Liu, Biophys J 81, 2395 (2001).
Global coefficient	When checked the current coefficient values are applied to all open image documents in **FRET View**.
Drop down selection	Choose which parameter set should be saved or loaded. Choose between **Acceptor** or **Donor**.
Save	Save the coefficients of **Donor** or **Acceptor** as .xml file.
Load	Load the coefficients of **Donor** or **Acceptor** for the current analysis.

13.5.2.11.3.3 Threshold tab

The threshold for image analysis can be set manually using the slider or editing text box next to **Donor**, **Acceptor**, **FRET** or **All**, where **All** moves all three sliders to the same value.

Alternatively, the threshold can be set by defining and enabling a background region in the **FRET** tab. The values for the thresholds are either displayed as grey value levels (Raw data) or Normalized to the value **1**.

Activate the general setting: Subtract Background using thresholds when background values should be subtracted before image analysis is performed.

Parameter	Description
Donor	Define threshold for analysis of donor signal.
Acceptor	Define threshold for analysis of acceptor signal.
All / FRET	Define threshold for analysis of all signals.
Clear	Set all threshold values to zero, deactivate background region.
Normalized	Grey values normalized to zero.
Raw data	Absolute grey values.

13.5.2.11.3.4 Settings tab

The tab allows to select parameters used for image analysis.

The **General** tab applies to both methods, **Acceptor Photobleaching** and **Sensitized Emission**.

Pixels of grey value zero are not included in any analysis.

13.5.2.11.3.4.1 General tab

Parameter	Description
Subtract back-ground using thresholds	The previously defined grey values of the thresholds (**FRET** tab, **Thresholds** tab) are subtracted from each pixel prior to analysis. If unchecked, pixels below the defined thresholds will not be displayed in the **FRET** Image and are not part of the analysis (value and area).
Exclude saturated pixels	Pixels which are saturated in at least one channel will not be considered for analysis.
Include thresh-olded pixels	Pixels with the same grey value as the defined thresholds will be part of analysis.
Apply palette	The **FRET** image is displayed color coded.
Show palette	Displays a palette of the **FRET** values within the **FRET** image and a palette of the channel colors in the raw data image.

13.5.2.11.3.4.2 Acceptor Photobleach tab

Parameter	Description
Do not show nega-tive values in Fc image	Does not display any negative values in the **FRET** image

13.5.2.11.3.4.3 Sensitized Emission tab

Parameter	Description
FRETN Truncation	Only includes the range of the selected values in result table and the displayed image

Parameter	Description
N-FRET Truncation	Only includes the range of the selected values in result table and the displayed image
Do not show negative values in Fc image	Does not display any negative values in the **FRET** image showing the analysis according to the method of Youvan.
FRETN normalization	Does not display any negative values in the **FRET** image showing the analysis according to the method of Youvan.

13.5.2.11.3.5 Analysis Methods for Sensitized Emission

13.5.2.11.3.5.1 FC or Youvan Method

Youvan et al., Biotechnology et alia 3, 1 (1997)

Fc = Ff-Df(Fd/Dd)-Af(Fa/Aa)

Displays the Fc image with intensities converted from the **FRET** index calculated for each pixel using the Youvan method. This method assumes that the signal recorded in the **FRET** channel is the sum of real **FRET** signal overlaid by donor crosstalk and acceptor signal induced by direct (donor) excitation. There is no correction for donor and acceptor concentration levels and as a result the **FRET** values tend to be higher for areas with higher intensities.

13.5.2.11.3.5.2 FRETN or Gordon Method

Gordon et al., Biophys J 74, 2702 (1998)

FRETN = FRET1/ Dfd * Afa

Displays the **FRET** image with intensities converted from the **FRET** index calculated for each pixel using the Gordon method. This method calculates a corrected **FRET** value and divides by concentration values for donor and acceptor. This method attempts to compensate for variances in fluorochrome concentrations but overdoes it. As a result, cells with higher molecular concentrations report lower **FRET** values.

13.5.2.11.3.5.3 N-FRET or Xia Method

Xia and Liu, Biophys J 81, 2395 (2001)

NFRET = FRET 1/ √(Dfd*Afa)

Displays an **N-FRET** image with intensities converted from the **FRET** index calculated for each pixel using the Xia method. This method is like the Gordon method with the difference that the square root of donor and acceptor concentration is used for concentration compensation. The resulting image is properly corrected for variances in the fluorochrome concentration.

The **Sensitized Emission** analysis (all three methods are analyzed) shows the following parameters in the data table beneath the **FRET** image and the original image:

Parameter	Description
Region	The identification numbers of regions assigned for analysis. Region zero refers to the whole image.

Parameter	Description
Time	When a time series is analyzed, the time point is shown.
D avg.	Average intensity of the region in the **Donor** channel. These values are influenced by the chosen settings in the **Settings** Tab. They may therefore vary from values of the same regions in the **Histo** tab.
A avg.	Average intensity of the region in the acceptor channel. These values are influenced by the chosen settings in the **Settings** Tab. They may therefore vary from values of the same regions in the **Histo** tab.
F avg.	Average intensity of the region in the **FRET** channel. These values are influenced by the chosen settings in the **Settings** Tab. They may therefore vary from values of the same regions in the **Histo** tab.
FRETN (p)	Result of analysis using Gordon method. The **FRETN** value is calculated for each pixel, afterwards the average for the chosen region is determined.
Fc (p)	Result of analysis using Youvan method. The **Fc** value is calculated for each pixel, afterwards the average for the chosen region is determined.
N-FRET (p)	Result of analysis using Xia method. The **N-FRET** value is calculated for each pixel, afterwards the average for the chosen region is determined.

13.5.2.11.4 Acceptor Photobleaching

13.5.2.11.4.1 FRET tab

Parameter	Description
Graphics tools	Use graphics tools to define the region(s) to be analyzed including the (optional) region which defines background signal.
Method	Select the analysis method. With an input of a dual channel time series with bleach event only **Acceptor Bleaching** analysis is possible.
Create Image	The displayed **FRET** image is displayed as new image document.
ROI table	Checkboxes assign the defined regions as region to be analyzed or background region.

The **Acceptor Photobleaching** analysis shows the following parameters in the data table beneath the **FRET** image and the original image:

Parameter	Description
Region	The identification number of regions assigned for analysis. Region zero refers to the whole image.
FRET(p) Eff. %	**FRET** efficiency for each pixel is calculated and averaged for all pixels of the region.
D Pre	The averaged intensities of the region are used to calculate an averaged **FRET** efficiency for this region. **Delta D** / **D Post** * 100.
D Post	Average donor intensity of the region in the pre-bleach image.

Parameter	Description
A Pre	Average acceptor intensity of the region in the pre-bleach image.
A Post	Average acceptor intensity of the region in the post-bleach image.
Delta D	Change in donor intensity of the region before and after the bleach event. **Delta D** = **D Post**-**D Pre**.
Delta A	Change in acceptor intensity of the region before and after the bleach event. **Delta A** = **A Post**- **A Pre**.

13.5.2.11.4.2 Parameter tab

Parameter	Description
Donor Ch	Define the channel which contains the **Donor** signal
Acceptor Ch	Define the channel which contains the **Acceptor** signal
Pre-bleach image: <nr>	Indicates the image before bleach event used for analysis
Pre-bleach image: <nr>	Indicates the image after bleach event used for analysis
Image after bleach event	In case of repetitive bleaching select the bleach event which should be analyzed. The post-bleach image number is updated accordingly.

13.5.2.11.4.3 Settings tab

The tab allows to select parameters used for image analysis.

The **General** tab applies to both methods, **Acceptor Photobleaching** and **Sensitized Emission**.

Pixels of grey value zero are not included in any analysis.

13.5.2.11.4.3.1 General tab

Parameter	Description
Subtract back-ground using thresholds	The previously defined grey values of the thresholds (**FRET** tab, **Thresholds** tab) are subtracted from each pixel prior to analysis. If unchecked, pixels below the defined thresholds will not be displayed in the **FRET** Image and are not part of the analysis (value and area).
Exclude saturated pixels	Pixels which are saturated in at least one channel will not be considered for analysis.
Include thresh-olded pixels	Pixels with the same grey value as the defined thresholds will be part of analysis.
Apply palette	The **FRET** image is displayed color coded.
Show palette	Displays a palette of the **FRET** values within the **FRET** image and a palette of the channel colors in the raw data image.

13.5.2.11.4.3.2 Acceptor Photobleach tab

Parameter	Description
Do not show negative values in Fc image	Does not display any negative values in the **FRET** image

13.5.2.11.4.3.3 Sensitized Emission tab

Parameter	Description
FRETN Truncation	Only includes the range of the selected values in result table and the displayed image
N-FRET Truncation	Only includes the range of the selected values in result table and the displayed image
Do not show negative values in Fc image	Does not display any negative values in the **FRET** image showing the analysis according to the method of Youvan.
FRETN normalization	Does not display any negative values in the **FRET** image showing the analysis according to the method of Youvan.

13.5.2.11.4.4 Threshold tab

The threshold for image analysis can be set manually using the slider or editing text box next to **Donor**, **Acceptor**, **FRET** or **All**, where **All** moves all three sliders to the same value.

Alternatively, the threshold can be set by defining and enabling a background region in the **FRET** tab. The values for the thresholds are either displayed as grey value levels (Raw data) or Normalized to the value **1**.

Activate the general setting: Subtract Background using thresholds when background values should be subtracted before image analysis is performed.

Parameter	Description
Donor	Define threshold for analysis of donor signal.
Acceptor	Define threshold for analysis of acceptor signal.
All / FRET	Define threshold for analysis of all signals.
Clear	Set all threshold values to zero, deactivate background region.
Normalized	Grey values normalized to zero.
Raw data	Absolute grey values.

13.5.3 General View Options

The general view options are visible in any view. Some of the view options are only visible when you open a particular file type. E.G. see the **ApoTome** view option only if you have opened a **ApoTome** image.

13.5.3.1 Dimensions Tab

Here you configure the settings for how the image will be displayed on the screen. You can select the size of the display and call up information about the content of the image. In the case of multidimensional images you can select here which dimension is displayed. The dimension sliders (e.g. time, channels) help you to navigate through the single images of an experiment.
Note: The displayed settings in the tab depend on the image and experiment. Also the settings of this tab in the 2D and 3D Correlative Workspace viewer are limited and/ or different compared to the normal ZEN image view.

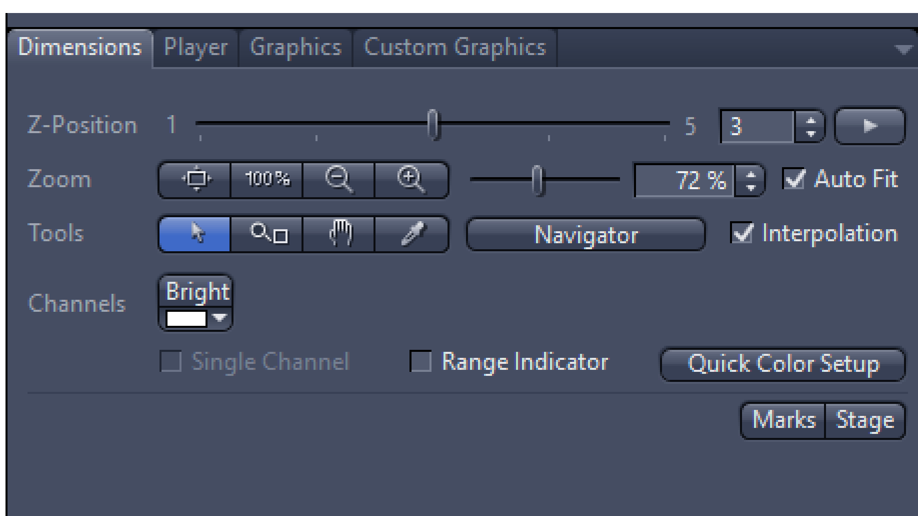


Fig. 79: Dimensions Tab (Show All)

13.5.3.1.1 Slider section

Depending on how many dimensions your image contains, several sliders can be available in this section. Using the sliders you can adjust the position that you want for each dimension available in the image. You will see the current position number in the input field to the right of the relevant slider. You can also enter the position number directly here.

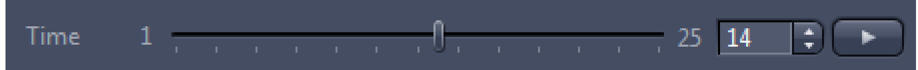


Fig. 80: Slider Section (example)

The [▶] **Play** buttons to the right of the input fields enable you to play back the dimension automatically. This takes place at a rate of 5 images per second by default. You can change the speed on the *Player Tab [▸ 893]*.

Info

For images with more than 3 dimensions a scrollbar is displayed which you can use to access the other sliders.

Depending on the available dimensions, the following sliders can be visible:

Parameter	Description
Follow Acquisition	By activating the **Follow Acquisition** checkbox during acquisition of multi dimensional experiments (time series, tiles and z-stacks) the actual image is displayed (default value). By moving the **Time** slider in the **Dimensions** tab, this feature is disabled and a button for performing a manual update of the slider range is shown.
Update	Only visible if **Follow Acquisition** is deactivated. Triggers a manual update of the acquired image during the running acquisition.
Z-Position	Only visible in the case of z-stack images. Here you can adjust the desired z-position.
X-Position	Only visible for images acquired in Line scan mode. Here you can adjust the desired x-position.
Time	Only visible in the case of time series images. Here you can adjust the desired time point.
Scene	Only visible if the image contains different scenes. Adjusts the desired scene. If you deactivate the **Scene** checkbox, all scenes are displayed in an overview.
Global-Z	Only visible for ZEN Connect projects with at least one z-stack. Sets the global value for the displayed z-slices. The range of the slider is defined by the z values of all stacks in the project. **Note:** When you use the **Global Z** slider and are beyond the range of a certain image, only a frame for this image is displayed to show where the image is positioned.
Block	Only visible if you have used the **Experiment Designer** and created several experiment blocks. Adjusts the desired experiment block.
Total Time	Only visible if you have used the **Experiment Designer**. Adjusts the duration across all blocks.
Phase	Only visible for ApoTome images and if on the *ApoTome tab [▸ 905]* the **Display Mode** is set to **Raw Data**. Selects the various phases of the raw images.

13.5.3.1.2 Zoom section

This section contains tools that you can use to adjust the size of the image region displayed.

Fig. 81: Zoom section

Info

If you hold down the *Ctrl*-key, you can zoom in and out of the image using the mouse wheel.

Parameter	Description	Short cut
Fit to View	Automatically sets a zoom factor at which the entire image can be displayed visibly on the screen.	*Ctrl + 0*
100% **Normal View (100%)**	Shows the image without increasing or decreasing the zoom factor. One pixel of the image corresponds to one pixel on your screen.	*Ctrl + 1*
Decrease Zoom	Decreases the zoom factor.	*F8, Ctrl + F8*
Increase Zoom	Increases the zoom factor.	*F7, Ctrl + F7*
36 % **Zoom factor**	Here you can set the display size steplessly. The desired zoom factor can be entered in the input field in percent.	
Auto-Fit **Auto-Fit**	**Activated:** Adjusts the display size automatically and continuously to the size of the window.	

13.5.3.1.3 Tools section

Fig. 82: Tools section

Parameter	Description
Selection	Activates the selection mode.
Zoom Rectangle	Activates the zoom mode. Hold down the left mouse button and drag out a selection rectangle. When you release the left mouse button, the region within the rectangle is displayed in enlarged form. If you have a mouse with a mouse wheel, you can also use this to move enlarged/reduce image regions.
Move	Activates the move mode. Left-click inside an enlarged image to move the zoomed region. If you have a mouse with a mouse wheel, position the mouse pointer inside the image region and hold down the mouse wheel. The mouse pointer will then appear automatically as a hand icon. You can now move the image region.
Inner Zoom	Only visible in **Gallery** view. This function allows you to set a new zoom level for all images simultaneously using the mouse wheel. The size of the Gallery view does not change here. This allows you to limit the view to one interesting image region. Use the **Move** tool to move the view.

Parameter	Description
Show Pixel Values	Activates the show values mode. If you move the mouse pointer into the image region, a vertical arrow and a display field will appear. The pixel values of the position to which the arrow is pointing are displayed in the display field. In the first line of the display field the X/Y coordinates are shown. The second line shows the X/Y coordinates in scaled units. In the other lines the gray values for each channel are shown.
Navigator	Opens the in the image area. There you will see an overview of your image and you can navigate to different positions using a rectangular window.
Interpolation	**Activated:** The pixel elements of the image are shown in an interpolated display. This makes it possible to avoid the pixelated display of small or greatly enlarged images. **Deactivated:** The pixel elements of the image are displayed as they are. This function is activated by default. You can deactivate this function via **Tools** menu | **Options** | *Documents Tab [▸ 633]*.

13.5.3.1.4 Channels section

This section contains all channels that you are using in your image. You can switch the display of channels in images on or off and change the channel colors (pseudo color assignment).

Fig. 83: Channels section

A button is displayed for each channel. Each button has two functions:

1. The channel name is displayed in the top section. To switch a channel off/on again, left-click on this section of the button.
2. The bottom section of the button shows the channel color. The display changes depending on the status of the button:

- When switched off, you will see a colored line below the button.
- When switched on, you will see a color field with a dropdown list below the button. Clicking on the dropdown list opens the color selection, see *Color Selection Dialog [▸ 892]*.

Info

For images with 8 or more channels, the **Channel** buttons are reduced in size. In this case it is no longer possible to change the color channel by channel.

Parameter	Description
Single-Channel	**Activated:** Only a single channel is displayed.
Show Channel Colors	Only visible in **Split** view. **Activated:** Displays the individual channels of multichannel images with the assignment of pseudo colors.

Parameter	Description
Range Indicator	**Activated:** Changes the display to single channel mode. The channel is displayed in monochrome. At the same time you will see pixels that are saturated (displayed in red) and pixels that have no signal (values = 0; displayed in blue). Note that with camera systems it is normally not possible to achieve pixel values of 0. The blue indicator is therefore normally not displayed. This function helps you to set your acquisition settings, camera exposure or detector gain, so that saturation of the detector is avoided. The range indicator function is not available for the sum channel of the Airyscan.
Quick Color Setup	Opens a dialog that allows you to select a color quickly for all channels of a multichannel image. The following options can be set:
- None	All channels are displayed without a pseudo color.
- Grayscale	All channels are displayed in monochrome (this applies in particular to multichannel images that have been acquired using color cameras).
- BGR	Channel 1: blue, channel 2: green, channel 3: red, no color assigned to any other channels.
- GRB	Channel 1: green, channel 2: red, channel 3: blue, no color assigned to any other channels.
- RGB	Channel 1: red, channel 2: green, channel 3: blue, no color assigned to any other channels.
- Via LUT	Colors for all channels are selected using a reference look-up table. The LUT is divided up into as many sections as there are channels, with the channel color being used at the separation point. You can select the reference LUT using the **Reference LUT...** button.
- Custom	The colors defined by the user are restored.
- Dye	The color of the dye used during the experiment is restored

On the bottom of the tab further controls are available:

Parameter	Description
Stage	**Activated:** You can move the stage with the mouse during a continuous acquisition by clicking and dragging the displayed red cross in the image container.
Marks	**Activated:** Stage coordinates (Marks) can be defined by clicking into the image. The coordinates are written into the Marks table in the **Stage** tool . The Marks button stays available during image acquisition
Selected Image	Only available in the ZEN Connect 3D Viewer. Selects the active image to which the settings or transformations should apply.

13.5.3.1.4.1 Color Selection Dialog

Here you can select a pseudo color for the selected channel. In the lower area of the dialog you will see four buttons that offer various methods of color selection. The selected button is highlighted in blue. To change the method, simply click on the appropriate button.

Fig. 84: Color Selection dialog

Parameter	Description
Weight	Sets the weighting of one channel to another channel. This is only possible with multi-channel recordings.
Color	Here you can choose the desired color from a default color chart. The selected color is displayed on the color button.
LUT	LUT = Look-Up Table Here you can choose the desired color from a more complex color look-up table.
Cust...	Cust = Custom Here you can define an own color and assign it to a color field.
None	Assigns no color to the channel. Images of monochrome cameras are black/white display. images of color cameras are displayed in real colors.

13.5.3.2 Player Tab

Only visible if the **Show All** mode is activated.

Using the functions on this tab you can play back multidimensional images. The functions largely correspond to the functions for playing back films.

Player Options

The following control elements are available:

Parameter	Description
Play	Plays back the image series forwards from first to last image. The dimensions are played back one after the other in the sequence specified.
Stop	Stops the play-back of the image series.
Play	Plays back the image series backwards from last to first image. The dimensions are played back one after the other in the sequence specified.
Play alternately	Plays back the image series forwards and backwards alternately.
Jump to first	Jumps to the start of the image series.
Jump to last	Jumps to the end of the image series.

Parameter	Description
Speed (FPS)	Here you can adjust the speed at which an image series is played back. The speed is displayed in frames per second (FPS) in the input field. You can also enter the desired speed directly in the input field. The maximum play-back speed is 25 FPS.
Follow Acquisition	**Activated:** Always displays the last acquired image during an ongoing acquisition procedure, as well as the slider for the corresponding dimension.
Dimensions	Depending on the available dimensionsin the active image a slider is displayed here for each dimension. Possible sliders: ▪ **Z-Stack** ▪ **Time** ▪ **Scene** ▪ **Block** The sliders have each two adjustment handles, which you can use to define the start and end point of the playback.

Parameter	Description
	If there are several dimensions, you can determine, by activating the corresponding checkbox, if you want the dimension to be taken into account during the play-back. Each slider offers as many steps as there are individual positions in the specified dimension. A third adjustment handle indicates the current position and cannot be controlled directly.

13.5.3.3 Graphics Tab

Here you can select various tools and use these to draw graphic elements into your images. In the list you see the graphic elements that you have drawn in to the image.

Global Graphics

In general there are two classes of graphic elements: global and custom. Each global graphic element has a set of properties such as style or type of measurement values displayed, which can be changed system wide for each element. Each global element can only have one formatting style which is used every time, this element is being used. All graphic elements can be accessed through the **Graphics** menu, a selection of the most important tools is also available in the **Graphics** view options tab for quick access (see image below). The content of the Graphics tab cannot be modified however.

Custom Graphics

Custom graphic elements are available in the **Custom Graphics** tab. Here it is possible to configure a collection of graphical elements according to personal preference. It is also possible to create multiple copies of the same tool type with different formatting styles and measurement values which is not possible for global graphic elements. For more information, see *Custom Graphics Tab [▸ 901]*.

Info

Graphic elements are characterized by their formatting style, can be annotated with a free text and can contain measurement values such as geometric or gray value measurements. Add free text by double clicking any graphic element and typing in the desired text.

Note: You can select the graphical elements and modify them. For more information, see *Editing graphical elements and measurements [▸ 899]*.

A selection of global graphic elements to work with are available to you here. For more tools, open the *Graphics [▸ 620]* menu.

Parameter	Description
Select	Use this to select the graphic elements in the image area. If you are currently in another mode, you can switch back to the Selection mode using this button.
Clone	Use this to create an identical copy of the last graphic element drawn in by simply clicking anywhere into the image area. To exit this mode, either switch back to the Selection mode or press the *ESC* key.

Parameter	Description
Draw Region of Interest	Use this to draw in a Region of Interest (ROI) into the image. ROI's are only used to create subsets of images, not for annotation of images. To create a subset, hold the *Ctrl* key pressed and move the mouse cursor within the ROI. Then press the left mouse key and wile keeping it pressed down drag the mouse outside the ROI. A new subset image document only containing the pixels within the ROI but with all other dimensions is being created.
Draw Text	Use this to insert a text field into the image. With the field drawn in start typing to add text.
Insert Scale Bar	Automatically inserts a scale bar into the bottom right corner of the image. The size is set automatically to approximately 5% of the width of the image size. The length can be modified by selecting the scale bar in the image and changing the length.
Draw Arrow	Use this to draw in an arrow.
Draw Rectangle	Use this to draw in a rectangle that is always parallel to the edges of the image. This element by default also shows the mean gray level of the image region.
Draw Circle	Use this to draw in a circle. This element by default also shows the mean gray level of the image region.
Draw Spline Contour	Use this to draw in a freely selectable contour. You can either define the corner points by a series of clicks or you can trace a contour by keeping the left mouse key pressed. Close this contour by right-clicking. Corners are always rounded with this tool. This element by default also shows the mean gray level of the image region.
Format	Only active, if you have selected a graphical element in the image or the **Annotations/Measurements** list. Opens the *Format Graphic Elements dialog [▸ 898]*. There you can format the selected graphic element according to your preference. Alternatively you can double-click on the list entry or right click on the graphical element in the image and select the **Format** entry.
Relative Time	Adds a text box to the top left of the image with information on the relative acquisition time per channel. Relative means, that the time value is set to 0 at the time point, where the element is drawn in. This makes it easier to analyze time series images.
Acquisition Time	Adds a text box to the top left of the image with information on the absolute acquisition time per channel.
Relative Focus	Adds a text box with information about the relative focus position. Relative means, that the focus value is set to 0 at the z-plane the element is drawn into. This makes it easier to interpret focus changes when playing through the image dimensions.
Focus Position	Adds a text box with information about the absolute focus position as recorded from the focus drive of the microscope.

Parameter	Description
Exposure Time	Adds a text box with information about the exposure time used by the camera given in the format „00.000“ [ss.msec].
Multi Channel Name	Adds a text box with the names of all the active channels.
Carrier Container Name	Adds a text box with information on the carrier container name. This is only useful for images acquired from multiple scenes using the Tiles module. Examples are multiwell plates. With this annotation an image can be related to the well it came from.
Keep Tool	**Activated:** Keeps the selected tool active. This allows you to draw in a number of the same elements one after the other.
Auto Color	This parameter is only visible if **Show All** is activated. **Activated:** Uses a new color for each element drawn in.
Snap to Pixel	This parameter is only visible if **Show All** is activated. **Activated:** Draws in the graphic elements in a way that connects them to the image pixels. If image-pixel precise measurements shall be done, this option must be used. If this option is not active, the graphic elements are drawn into the graphics layer independently of the actual image pixels.

Layers

Only visible if the **Show All** mode is activated.

Here you can specify which graphic element layers are active and visible in the image. To open the shortcut menu, click on the Layers dropdown menu.

Parameter	Description
Active Layer	Here you can specify which graphics layer is active in the image. The other layers are visible but blocked. To activate a layer click on the menu entry.
- automatic	Sets the active layer automatically. This is the default setting.
- Selection	Sets the Selection layer as the active layer. This layer contains graphic elements such as ROI selection, Grid, etc.
- Annotations/ Measurements	Sets the Annotation/Measurement layer as the active layer. This layer contains most of the graphic elements which can be drawn in such as all annotation elements or interactive measurement tools.
- Acquisition	Acquisition elements are elements which have been used in experiments to specify acquisition ROI’s or photomanipulation ROI’s such as used for FRAP experiments.
Layers	Here you can specify which layer is visible in the image. The other layers are not visible.
- Selection	Displays the Selection layer in the image.

Parameter	Description
- Annotations/ Measurements	Displays the Annotation/Measurement layer in the image.

Table

Here you can see almost all the graphic elements that exist in your image. You can also control the behavior of the graphic elements here, e.g. lock or hide them. You can format each graphic element as you wish.

Info

In the list you will only see the graphic elements relating to the active graphics plane. To change the active graphics plane, click on the **Layers** button. This button is only visible in **Show All** mode. Select the layer that you want to display under **Active Layer**.

The columns of the list contain the following entries:

Parameter	Description
Visibility	Shows or hides a graphic element.
Fix Position	Locks a graphic element to prevent changes being made.
Type	Displays the icon for the tool type. To format a graphic element, double-click on the icon. The Format Graphic Elements dialog then opens.
ID	The parameter is only visible if **Show All** is activated. Displays the **ID** for the graphic element. To do this, activate the checkbox at the corresponding list entry. Note that each graphic element has its unique ID.
A	The parameter is only visible if **Show All** is activated. Displays **Annotations** for a graphic element. To do this, activate the checkbox at the corresponding list entry. „Annotations“ refers to all user defined text as well as text from frequent annotations.
M	The parameter is only visible if **Show All** is activated. Displays **Measurement** data for a graphic element. To do this, activate the checkbox at the corresponding list entry.
Name	Displays the name of the graphic element. To change the name, double-click in the Name field. Then enter the text of your choice. This can be used to label elements in your image.
Save	Saves the selected graphic element for reusing it with other images.
Load	Loads an existing graphic element into the current image.

Parameter	Description
Delete	Deletes the selected graphic element.

Dimension

The coordinates and dimensions of the selected graphic element are displayed in the corresponding input fields (standard unit = image pixels):

Parameter	Description
Scaled µm	**Activated:** The dimensions are shown in scaled unit.
X	Displays the X coordinate of the center point of a graphic element. Edit the X coordinate here.
Y	Displays the Y coordinate of the center point of a graphic element. Edit the Y coordinate here.
W	Displays the width of graphic elements. Change the width here.
H	Displays the height of graphic elements. Change the height here.
Angle	Displays the angle of rotation of graphic elements. Here the measured angle is displayed for the graphic element Angle. Change the angle here.

See also

Adding Annotations to Images or Movies [61]

13.5.3.3.1 Format Graphical Elements Dialog

This dialog can be called up via the menu **Graphics** |**Format** or via the **Graphics** view option tab.

Note that for the **Draw Region of Interest** function, a reduced amount of functionalities is available.

Parameter	Description
Zoom with image	If activated, the size of the graphic element (e.g. line width, given in nr. of pixels) is related to the pixel size in the image. Therefore, when zooming into the image, the line width increases in the same way as the image pixels. If deactivated, the size relates to the monitor pixel size. That means when zooming into the image, the line width e.g. does not change.
Line	Here you change the line color, width and the line style (none, solid, dashed and dotted).

Parameter	Description
Text	Here you select the text font, color, size and style by selecting the appropriate options. Also select the desired horizontal and vertical alignment from the dropdown list. Reading direction is only active for measurement annotations of the **Line** element. **One direction** aligns the text annotation to the edge of the image. **Two directions** aligns it parallel to the line itself.
Fill	Here you can adjust, if the background of the annotation should be filled or not. Several filling options are available.
Opacity	Here you can adjust the degree of opacity of the graphic element in percent. 100% makes the graphical element completely opaque (covering the underlying image pixels), while 0% makes it completely transparent.
Annotation	Here you can change the selected annotation and add the unit.
Set as new global default	If you click on this button the formatting style of the graphic elements currently selected in the image is set to the new default.
Reset	If you click on this button the formatting style of the currently selected graphic element is reset to factory default.

See also

Graphics Tab [894]

Adding Annotations to Images or Movies [61]

13.5.3.3.2 Editing graphical elements and measurements

You can change the following properties of a graphical element or an interactive measurement:

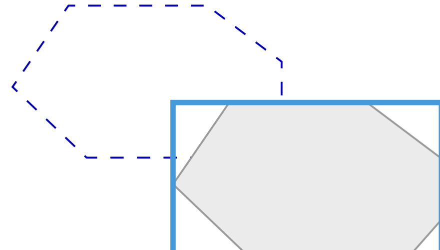
- Size and position of entire element.
- Position of measurement result.

You can also copy, paste, and delete elements and for some tools you can change the orientation.

Moving an entire element / measurement

Prerequisite ✓ At least one element is visible in the **Center Screen Area**.

1. Select the element with the left mouse button and drag and drop it to the desired position.



Resizing an entire element / measurement

Prerequisite ✓ At least one element is visible in the **Center Screen Area**.

1. Click on the respective element.
2. Drag the sides of the bounding box.

Copying / pasting an element:

Prerequisite ✓ At least one element is visible in the **Center Screen Area**.

1. Click on the respective element.
2. Press *Ctrl+C* to copy or *Ctrl+V* to paste the selected element. Alternatively, right-click the element and select **Copy** or **Paste**.

Rotating an entire element / measurement

Prerequisite ✓ At least one element is visible in the **Center Screen Area**.

1. Click on the respective element.
2. Select the node above the element wit the left mouse button and rotate it to the desired position.

Deleting an entire element / measurement

Prerequisite ✓ At least one measurement is visible in the **Center Screen Area**.

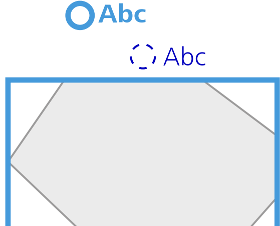
1. Click on the respective element.
2. Press the *Del* key. Alternatively, right-click the element and select **Delete**

Moving the measurement result

To change the properties of the measurement result:

Prerequisite ✓ At least one measurement is visible in the **Center Screen Area**.

1. Select the node by the measurement result with the left mouse button and drag it to the desired position. Note that the node can be hard to differentiate from the bounding box of the element.



13.5.3.4 Custom Graphics Tab

Only visible if the **Show All** mode is activated.

Here you can create your own preferred collection of graphic elements. You can add multiple instances of the same element but assign different formats or measurement values to them. Custom graphics should not be confused with the standard graphic elements the formatting style of which can only be changed globally (meaning one default style per element is used system wide). When opened for the first time, no elements exist. Click **Customize** to add your own graphic elements.

Parameter	Description
Select	Use this to select the graphic elements in the image area. If you are currently in another mode, you can switch back to the Selection mode using this button.
Clone	Use this to create an identical copy of the last graphic element drawn in by simply clicking anywhere into the image area. To exit this mode, either switch back to the Selection mode or press the *ESC* key.
Customize	Opens the **Customize Tools** dialog. In this dialog you can add up to 35 graphic elements which are organized in up to 5 tool bars. You can make changes to their formatting style and also define which measurement values they should show.
Keep Tool	**Activated:** Keeps the selected tool active. This allows you to draw in a number of the same elements one after the other.
Auto Color	This parameter is only visible if **Show All** is activated. **Activated:** Uses a new color for each element drawn in.

13.5.3.4.1 Customize Tools Dialog

This dialog is called up via the **Custom Graphics** view option tab.

Parameter	Description
User Toolbar	This list shows all custom graphic elements added to the **Custom Graphics** tab. Select a tool and double click on it’s icon to open the **Format New Custom Graphic Tool** dialog, see *here [▸ 898]*. You can rearrange the order of elements by using the **Up** or **Down** arrows at the bottom edge of the list. To delete a selected element use the **Delete** icon at the bottom edge of the list.
Search	Type in text to search for specific graphical elements.
Tools	This list contains all graphical elements available. If you select an element and double click on it, it will be added as a new entry to the top row of the **User Toolbar** list. Alternatively the Button with the Plus symbol at the bottom edge of the list can be used.

Parameter	Description
Frequent Annotations	This list contains all frequent annotations available. Frequent annotations are aligned rectangle elements preconfigured to show image metadata such as acquisition time or focus position used during acquisition. If you select an element and double click on it, it will be added as a new entry to the top row of the **User Toolbar** list. When finished, click the **Close** button. The newly added elements are now shown in the **Custom Graphics** tab.

13.5.3.5 Display Tab

Here you can adjust the image display. This function is particularly important if you want to display images with a very high dynamic range on the screen.

The histogram shows the brightness distribution of the pixels that are present from all channels simultaneously. The y-axis represents the relative frequency and the x-axis indicates the brightness. A curve showing the corresponding distribution, the so-called display characteristic curve, is displayed for each channel.

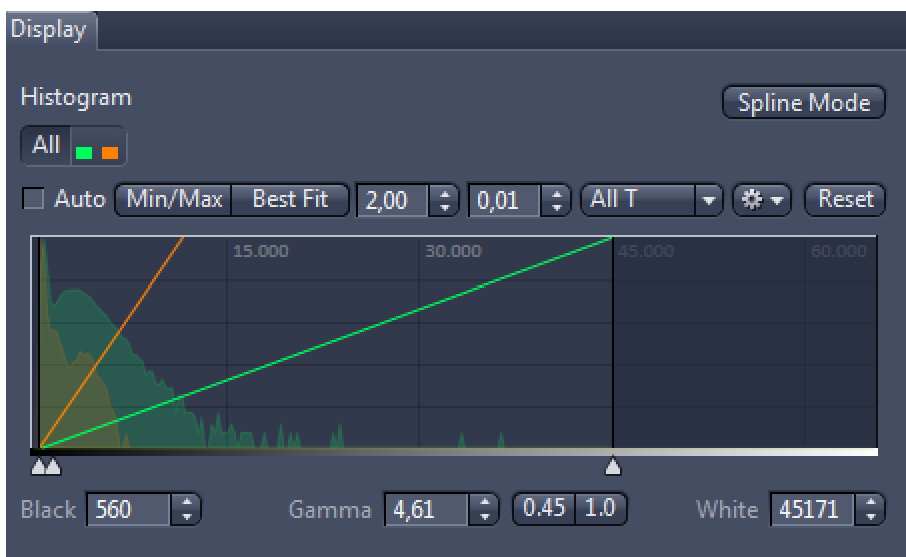


Fig. 85: Display tab (Show All)

If you want to adjust the histogram for an individual channel, activate this via the relevant color field in the **Channel Selection**. Alternatively you can also click on the corresponding distribution curve directly in the histogram.

Display characteristic curve

Each channel has a display characteristic curve. Using the display characteristic curve you can set the limit for the black value (left) and the limit for the white value (right). This allows you to influence the contrast in the image. Move the mouse pointer over the corresponding adjustment handles at the bottom edge of the display histogram or to the small rectangles on the display characteristic curve. Hold down the left mouse button and move the adjustment handles or rectangles as required.

The curvature of the display characteristic curve influences the gamma value. To change the curvature, move the mouse pointer to the second or fourth small rectangle on the display characteristic curve. Hold down the left mouse button and move the rectangles up or down. The setting is used immediately for the display. Using the middle rectangle you can move the whole display curve. This changes the brightness of the image.

Note that some of the functionality is only visible if the **Show All** mode is activated!

Parameter	Description
All **Channel Selection**	Only visible for multichannel images. Here you can select for which channel you want to adjust the display on the screen. To select all channels, click on the **All** button. To select a certain channel, click on the corresponding channel field. Hovering the mouse pointer over a color field displays the relevant channel name. If the image consists of more than 29 channels, a scrollbar will be displayed which you can use to switch to the desired channel.
Spline Mode	Clicking on this button allows you to add up to 8 points to the display characteristic curve. You can then bend the curve around these points. To do this, click on the desired section of the display curve and move it as required. Clicking on the display curve again adds another point. You can delete points by moving them along the display curve until they lie on top of another point. In this way, even in difficult situations you can adjust the display curve so that all important image regions can be displayed well.
Auto	The automatic adjustment is not available during the acquisition of LSM experiments. Adjusts the image display automatically. This is particularly useful in the case of a live image, in **Continuous** mode or if you play back a time series image that contains changing brightness.
Min/Max	Adjusts the display characteristic curve so that the darkest pixel is black and the brightest pixel is white in the display. Note that this function makes an approximation to get a quick result! For detailed information about minimum and maximum values refer to measurements conducted in the **Histo** view.
Best Fit	Adjusts the display characteristic curve so that 0.1% of the darkest pixels contained in the image are black and 0.1% of the brightest pixels are white in the display.
Input fields	With the two input fields to the right of the **BestFit** button you can adjust the black/white values from 0.1% to values from 0 to 90% according to your requirements.
Dimension Selection	If your images contain time series, z-stacks or both, you can here select the aspect of an image for which the display settings should be applied. Note that with all settings other than **Current** there may be several seconds of calculation time until the setting is applied, depending on the number of time points/ z-planes. The following options are available:

Parameter	Description
- Current	Adjusts the display for the current image and keeps this setting for all other time points or Z-planes.
- All T	Collects the intensity values from all time points and adjusts the display according to the brightest and darkest pixels within the entire time series.
- All Z	Collects the intensity values from all Z-planes and adjusts the display according to the brightest and darkest pixels within the entire Z-stack.
- All T+Z	Collects the intensity values from all Z-planes and time points and adjusts the display according to the brightest and darkest pixels within the entire Z+T series.
Options	Here you can copy display settings to the clipboard, insert them into other images from there or save and reload settings. This allows you to apply identical display settings to several images in order to produce comparable display conditions.
Reset	Resets all parameters to the default values.
Black	Displays the gray value currently set up to which all pixels are shown as black. You can also enter a certain value here.
Gamma	Displays the gamma value currently set. You can also enter a certain value here.
0.45	Sets a gamma value of 0.45. This is the recommended setting for most color images.
1.0	Sets a linear display characteristic curve with a gamma value of 1.0.
White	Displays the gray value currently set from which all pixels are shown as white. You can also enter a certain value here.

13.5.3.6 PSF tab

In most image views you will see the **PSF Display** tab as soon as a PSF image has been loaded. PSF images differ from the data types of normal images. They are saved, for example, in the high-precision floating point format. A series of important values that allow conclusions to be drawn about the microscope system and sample conditions can also be read from PSF images.

Export PSF button

Generates a new PSF document in 16 bit gray level format, which can be processed as required to allow it to be used in other programs.

Display Mode settings

Choose between three display options from the dropdown list:

- **Intensity PSF**: The PSF is displayed in the position space, gray values are displayed in floating point format.
- **Intensity OTF**: The optical transfer function (OTF) displays the 3D PSF in the frequency space following a 3D Fourier transformation. Gray values are displayed in floating point format.
- **Intensity Slice OTF**: Displays the 2D Fourier transformation of each individual Z-plane.

Axial cut view checkbox

Activated: Displays the PSF in axial section view.

Deactivated: A slider for Z appears on the Dimensions tab. This allows you to move through the various Z-planes.

List of PSF values

A series of important values relating to the PSF are displayed here in a table:

- **Storage type:** Format in which the PSF is saved
- **Source:** Shows whether the PSF has been generated by measuring a bead stack (External) or from the theoretical calculation (Internal).
- **Used Dimensions:** shows whether the PSF is 3D or 2D.
- **Instrument:** shows the type of microscope used.
- **Illumination:** shows the illumination conditions that applied.
- **NA Objective:** Numerical aperture of the objective
- **Lateral Magnification:** shows the objective magnification.
- **Working Distance:** shows the working distance of the objective.
- **Illumination Wavelength:** shows the wavelength of the excitation light; in the case of multichannel PSFs the values for all channels are shown here.
- **Detection Wavelength:** shows the wavelength of the detected emission light; in the case of multichannel PSFs the values for all channels are shown here.
- **Transverse Resolution (Rayleigh)**: shows the actual lateral resolution achieved according to Rayleigh; in the case of multichannel PSFs the values for all channels are shown here.
- **Axial Resolution:** shows the actual axial resolution achieved, determined according to Full Width Half Maximum (FWHM); in the case of multichannel PSFs the values for all channels are shown here.

Info

Please note that the resolution values for measured PSFs show the performance of the entire system, consisting of all optical and electronic components. The sample, with its optical properties and possible aberrations, therefore has a significant impact on the resolution. This means that these values are not suitable for making statements about the quality of the objective.

13.5.3.7 ApoTome tab

Here you have various settings for displaying an ApoTome image.

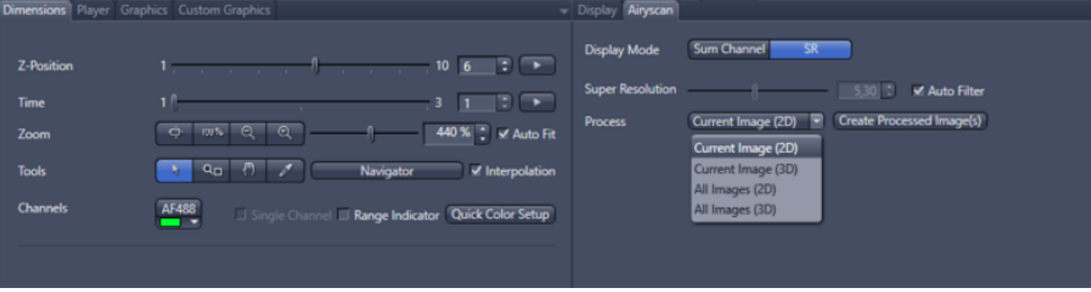
Parameter	Description
Display Mode	ApoTome images are acquired as raw data. The **Display Mode** sets how the image is calculated and displayed.
– Optical sectioning	The displayed image is calculated/rendered by eliminating the excitation and emission light that originates in regions outside of the focal plane.
– Conventional fluorescence	The displayed image is calculated/rendered like a conventional fluorescence image.

Parameter	Description
– Raw Data	Displays the raw data as output image and disables all other parameters of the function. Displays the **Phases** slider on the **Dimensions** tab. This enables you to select the various phases of the raw images.
Normalization	**Activated:** The resulting images always fill the entire 16 bit dynamic range of the image histogram, see normalization .
Enable Correction	**Activated:** Displays the **Correction** dropdown list for stripe artifact correction.
Correction	Only visible if **Enable Correction** is activated. Applies stripe artifact correction to the resulting image. It attempts to remove stripe artifacts which may be caused by bleaching of the sample during acquisition or by slight deviations in the grid phase position.
– Phase Errors	Corrects phase errors in the image without additional bleaching correction.
– Local Bleaching	Corrects the bleaching for each pixel individually (default setting). This is usually the best method.
– Global Bleaching	Corrects bleaching by means of global bleaching correction, which is applied equally to the entire image.
Phase Correction	Only visible if **Enable Correction** is activated and if you have selected one of the two bleaching corrections for **Correction**. **Activated**: Performs a correction of any phase deviations present in addition to the selected bleaching correction.
Fourier Filter	The Fourier filter attempts to remove residual stripes.
– Off	Uses no Fourier filter to remove stripes.
– Weak	Uses a weak Fourier filter to remove stripes.
– Medium	Uses a medium Fourier filter to remove stripes.
– Strong	Uses a strong Fourier filter to remove stripes.
Grid	Displays the grid frequency used for the image in lines/mm.
Deconvolution	**Activated:** Displays key parameters for **ApoTome** deconvolution. Deconvolution benefits from the presence of a CUDA enabled NVIDIA graphic card by speeding up the processing.
Aberration Correction	Only visible if **Deconvolution** is activated. **Activated:** Displays parameters for aberration correction.
– Refractive Index Embedding	Sets the refractive index of the used embedding medium .
– Distance to Coverslip	Sets the distance of the acquired structure from the side of the cover slip facing the embedding medium.

Parameter	Description
Set Strength Manually	Only visible if **Deconvolution** is activated. **Activated:** Sets the degree of restoration with the slider. To achieve strong image restoration, move the slider towards **Strong**. To achieve less image restoration, move the slider towards **Weak**. **Note:** If the setting is too strong, image noise may be intensified and other artifacts, such as "ringing", may appear. **Deactivated:** The restoration strength for optimum image quality is determined automatically. The restoration strength is inversely proportional to the strength of so-called regularization. This is determined automatically with the help of Generalized Cross Validation (GCV).
Apply Deconvolution	Only visible if **Deconvolution** is activated. Applies the deconvolution to the ApoTome image. The result is displayed directly without a new document being created. To create a separate document, click on the **Create Image** button.
Create Image	Creates a new image document. The available settings are taken into consideration here.

13.5.3.8 Airyscan tab

During acquisition of Airyscan 2 Multiplex mode images, the result can be displayed and controlled for correct acquisition quality with the **Airyscan** tab. **Note:** If you use multi-immersion objectives, the immersion medium needs to be indicated with the MTB software to allow for correct processing of Airyscan images.



Parameter	Description
Display Mode	
– Preview	You can see a more instantaneous refresh especially during continuous scan. This allows best control of focus and stage positions.
– SR	Shows a processing preview which displays a true super resolution processing of the current image content.
Super Resolution	Modifies the processing strength.
Auto Filter	Sets the processing strength automatically.

Parameter	Description
Process	In the drop down list you can select the image(s) and dimension you want to save. Available options: ▪ Current Image (2D) ▪ Current Image (3D) ▪ All Images (2D)
Create Processed Image(s)	Processes the active images and saves them.

13.6 View Modes

13.6.1 Full Screen mode

In this mode the image will be displayed in the full monitor size.

To start the full screen mode, position the cursor on the image area and open the context menu via right mouse click. Click on **Full Screen**. You can also press **F11** or click on menu **Window | Full screen** as an alternative.

Toolbar

In the toolbar at the bottom you find several buttons for general and image specific functions, like zoom function (**Zoom** button) or image informations (**Info** button). When you open a multidimensional image, you find buttons for specific functions, etc. **Z-Stack**, **Channels**. To open the functions, click on the button.

Previous button

Displays the previous document in full screen mode. You can page step by step backwards through all open documents.

Next button

Displays the next document in full screen mode. You can page step by step forwards through all open documents.

Exit Fullscr. button

Closes the full screen mode.

13.6.2 Exposé mode

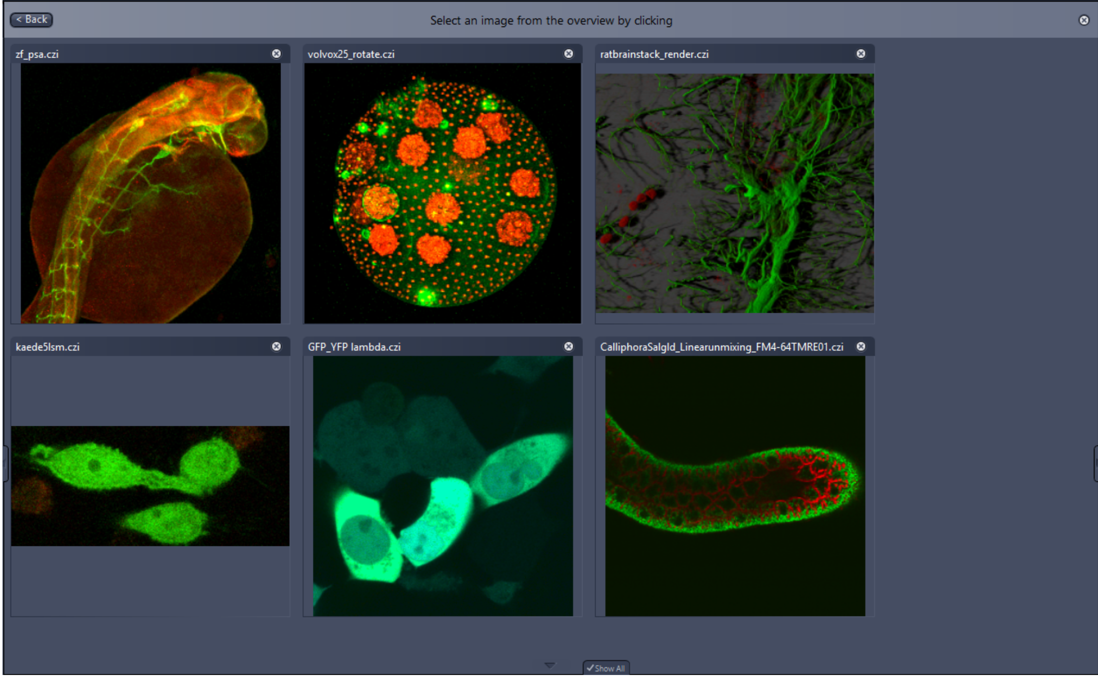


Fig. 86: Exposé mode

To open the **Exposé** mode click on the expose mode icon in the *document bar [▸ 22]*.
In this mode all open documents will be displayed in an overview. When you click on an image in the overview the **Exposé** mode will be closed and the image will be displayed in standard view. Click on **Back** to go back to the standard image view.

13.6.3 Splitter mode

To open the **Splitter** mode click on the splitter mode icon in the *document bar [▸ 22]*.
In this mode you can generate a multi image of one or several images in order to compare them. Drag an image of the **Images and Documents** gallery in **Right Tool Area** and drop it in a splitter position. The standard setting for the splitter are 2 columns and 1 row. You can modify these setting in the **Split-View**.
Proceed similarly with further images to be displayed in the multi image. The same image can be dropped several times in the splitter view, i.e. to compare different image scenes.
The multi image can be saved as **CZSPL** (Zeiss Multi Image Files) image type in the menu **File > Save As**. The stored multi image is no image document, but rather a reference of the images displayed in the splitter mode.
Use the **Split Display** tab for further adjustments (i.e. arrangement) of the splitter mode. Here you can create a single image of the multi image to be saved as **CZI** image file.

13.6.3.1 Split Display Tab

Arrangement section

Here you can set how much columns and rows the splitter image should have. Therefore simply enter the desired number in the **Columns** / **Rows** input fields.

Parameter	Description
Arrangement	

Parameter	Description
– Columns	Sets the number of columns displayed in the splitter view mode.
– Rows	Sets the number of rows displayed in the splitter view mode.
Synchronize Dimensions	**Activated:** The settings of the **Dimensions** tab (i.e. Zoom) will be applied synchronously to all images in splitter mode.
Synchronize Display	**Activated:** The settings of the **Display** tab (i.e. Gamma) will be applied synchronously to all images in splitter mode.
Show Position Data	**Activated:** The cursor changes to an arrow symbol and a cross marker in the image. The **X/Ycoordinates** with scaling unit and **gray value** of the current cursor position are displayed below the image. Furthermore additional information is displayed for multidimensional images: i.e. the **gray value for each channel** of a multichannel image, the **time of each time point** of a time lapse image, the **focus position of each Z position** of a Z-Stack.
Reset	By clicking on this button you can reset all adjustments applied to the images in splitter view.
New Image From	Here you can select the type of image to be generated. The available options are depending on the dimensions of the displayed image.
- Current View	Creates a 2-dimensional image of all opened images visible in the splitter mode.
- Time Series	Creates a multi-dimensional image containing each time point of a time series image.
- Z-Stack	Creates a multi-dimensional image containing each Z-plane of a Z-stack image.
- Rotation	Creates a multi-dimensional image containing each rotation plane of a rotation series image.
Create	Generates an single image of the multi image displayed in splitter mode with the corresponding settings. The image can be saved in the ***.CZI** format in **File > Save as** menu.
Interpolation for zoomed images	Only visible if the **Show All** mode is activated. Here you can select how you want interpolation to be performed if a pixel is calculated from several individual pixels.
- Nearest Neighbor	The output pixel is given the gray value of the input pixel that is closest to it.
- Linear	The output pixel is given the gray value resulting from the linear combination of the input pixels closest to it.
- Cubic	The output pixel is given the gray value resulting from a polynomial function of the input pixels closest to it.
Spacing	Only visible if the **Show All** mode is activated. Here you can adapt the size of the distance between the single images to values 0 up to 20. The background (distance) will be displayed in black color and appears, if the splitter image is generated by the **Create** button.

Parameter	Description
Spacing color	Only visible if the **Show All** mode is activated. Here you can change the background color of the splitter image.
Burn-in annotations	Only visible if the **Show All** mode is activated. **Activated**: All existing annotations and graphical elements within the images will be burned into the resulting splitter image.

13.7 File Browser

Here you see an overview of all image or data files stored on your computer. In the left column you see a file structure which is associated with the common image or data containing folders in your file system (Images and documents). In the right area you see the preview to the selected folder.

Info

The ZEN folders contain automatically the **Auto Save** folder. Here you see all auto-saved images from ZEN. Set Auto Save path in **Tools** | **Options** | **Saving**.

Gallery View

Here you see all files of a folder as small preview images (thumbnails). Use **Tool** tab to adjust preview images size, sorting, etc..

Info View

Here you see a detailed list with all data the selected image contains. Find a detailed description of all possible data under *Info View [825]*.

Table View

Here you see all files of a folder well-arranged in a table. This view is perfect for folders which contain many files.

13.7.1 Tools Tab

Parameter	Description
Icon size	Set size of thumbnail images here.
Text Rows	Select entries which you want to have displayed as additional text row under the thumbnail image.
Record	Switch from file to file in the selected folder by using the slider.
Sorting	Arrange your files to certain properties (i.e. file name, type, etc.)
Folders	Manage selected file folders here (i.e. new folder, rename folder, etc.).
Selection	Manage selected files here (i.e. copy file, or delete file).

14 Systems, Applications & Components

14.1 LSM 980

14.1.1 Polarization Imaging

Linear (plane) polarized light, which is light whose wave goes only one direction, exciting a fluorescent molecule with a preferred dipole orientation results in polarized emitted light. It provides a contrast-enhancing method that is especially useful in the study of molecules that are fixed in their orientation or are greatly restricted in their rotational diffusion. Anisotropy is directly related to polarization and is defined as the ratio of the polarized light component intensity to the total light intensity.

In polarization microscopy using LSM systems the sample is irradiated with vertical polarized light (in respect to the optical table) from a laser source. The emitted fluorescence is passed sequentially through emission polarizers (analyzers) that are positioned before the Quasar detector. They transmit either the vertical (I_{VV}) or horizontal (I_{VH}) polarized emitted light onto the Quasar detector (L format fluorescence polarization). Since the vertical component of the emission light is parallel polarized to the vertical polarized excitation light, it is often also referred to as the parallel component (P Pol, I_p, $I_{\|}$). Likewise, the horizontal polarized emission light is also designated as the perpendicular ("senkrecht" in German) component (S Pol, I_S, $I_{|}$). In the software the "p" and "s" designations are used.

Polarization **P** and anisotropy Polarisation P and anisotropy r are defined as: are defined as:

$$P = \frac{I_p - I_s}{I_p + I_s} \quad \text{and} \quad r = \frac{I_p - I_s}{I_p + 2 \cdot I_s} \quad \text{with } 0 \leq r \leq 1.$$

They can be interconverted to each other in the following way:

$$P = \frac{3 \cdot r}{2 + r} \quad \text{and} \quad r = \frac{2 \cdot P}{3 + P}$$

In a completely polarized sample ($I_S = 0$) the anisotropy r = 1. In a completely non-polarized sample ($I_s = I_p$) anisotropy r = 0.

The formulas for polarization **P** and anisotropy **r** as given above are strictly true only, if the optical transmission for both emission polarizers are identical. Any differences must be corrected by introducing a correction factor **G** that is multiplied with I_p. Hence the anisotropy **r** in such a case is calculated according to:

$$r = \frac{I_p - G \cdot I_s}{I_p + 2 \cdot G \cdot I_s}$$

G can be measured using horizontally polarized excitation light and is defined as

$$G = \frac{I_{HV}}{I_{HH}}$$

However, since in LSM systems the polarization of the excitation light can not be changed from vertical to horizontal, G has to be determined with an isotropic fluorescent dye solution as the ratio between the mean intensities I_p and I_s, e.g. obtained from the histogram view.

Info

Please note that the G factor is not the mean intensity of the ratio calculation (R), where every pixel is computed separately. It has to be calculated from the ratio of the mean intensities of the I_p and I_s images.

As the formulas implies:

Anisotropy r is the preferred display as anisotropy of single species is additive. The calculation of r can be done using ratio formulas located in the MeanRoi View.

14.1.1.1 Setting up Polarization Imaging

Prerequisite ✓ S and P Pol filters are part of the system configuration.

1. Set up a multitrack configuration using exactly the same settings with the exception of the Pol Anisotropy filters.
2. Click the **Pol Anisotropy filter wheel** located under the channel selection list.
   → This opens up the **Pol Anisotropy** selection panel.
3. Check the anisotropy filter you want to use for Track 1.
4. Switch to Track 2. Select the anisotropy filter you want to use for Track 2.
5. The options are **Pol Anisotropy P Filter** (for the parallel polarized light transmission) and **Pol Anisotropy S Filter** (for vertical polarized light transmission) (see picture).

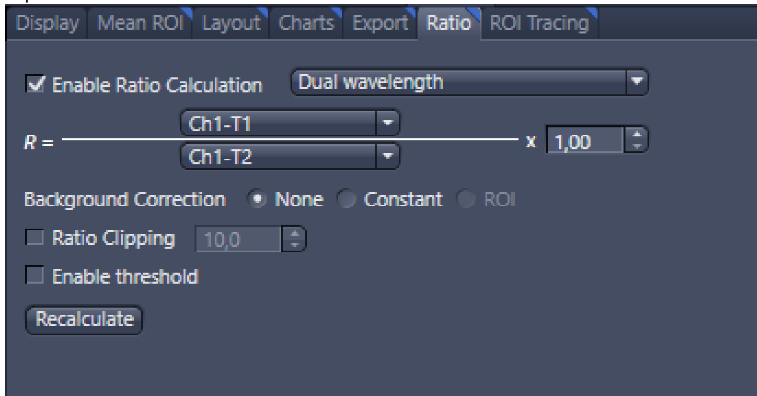
Info

If you want to calculate Anisotropy using the ratio imaging formulas provided by ZEN, then choose **P** for Track 1 and **S** for Track 2.

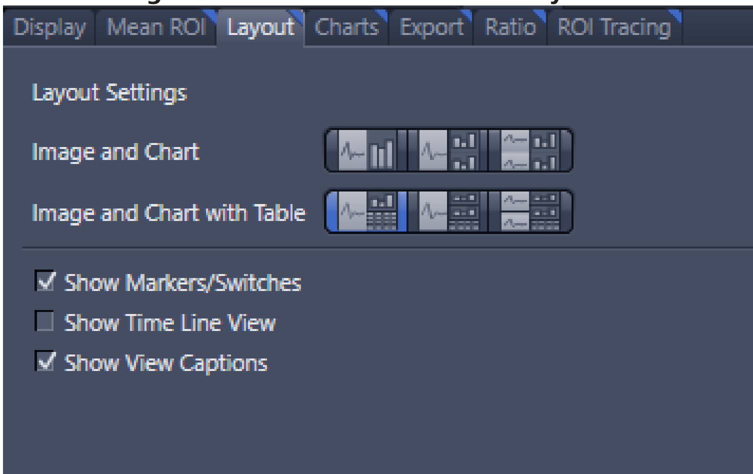
14.1.1.2 Determining the G Factor

Prerequisite
- ✓ Isotropic sample like a fluorescent dye at a 1 mM concentration, for example fluorescein.
- ✓ Multitrack image acquisition set as described above (Imaging Set up for Polarization Imaging) with excitation laser and detection beam path set for imaging the isotropic dye sample.
- ✓ Excitation laser intensity and detector gain set to get a well balanced image without overexposed pixels.
- ✓ **Pol Anisotropy P Filter** selected for Track 1 and **Pol Anisotropy P Filter** selected for Track 2.

1. Acquire a time series multitrack image with the image in Track 1 showing the emission signal filtered by the P Anisotropy filter and the image in Track 2 showing the emission signal filtered by the S Anisotropy filter.
2. Open MeanRoi View .
3. Open **Ratio** action tab.



4. Activate **Enable Ratio Calculation**.
5. Chose **Dual Wavelength** as method for ratio calculation.
6. Set factor to **1,00**.
7. Draw a (large) region into the image for analysis.
8. Activate **Image and Chart with Table** in the **Layout** action tab.



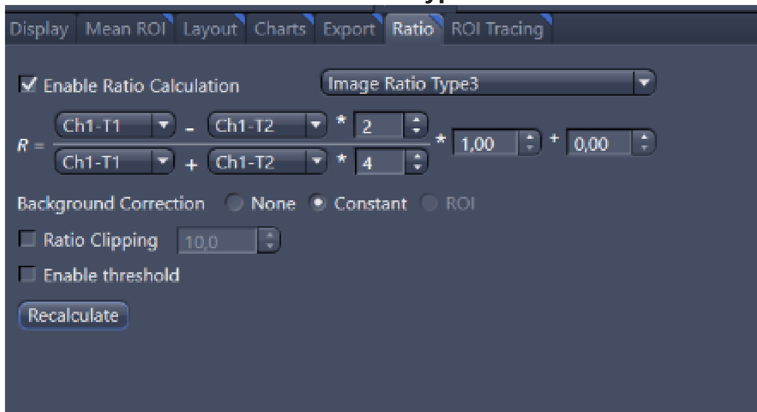
→ The G factor is shown as mean intensity value of the Ratio_R1 channel in the intensity chart.

The G-factor is therefore defined as:

$$G = \frac{Ch1 - T1}{Ch1 - T2} = \frac{I_P}{I_S}$$

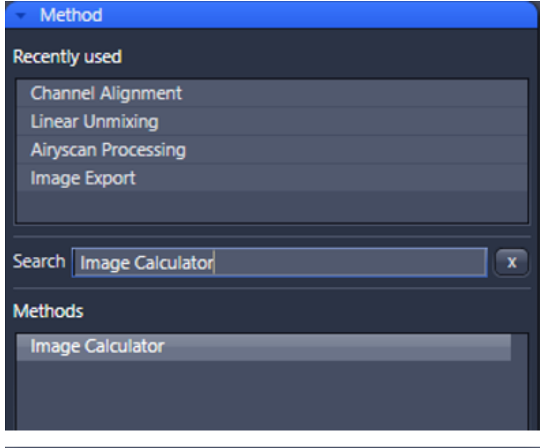
14.1.1.3 Obtaining an Anisotropy Image

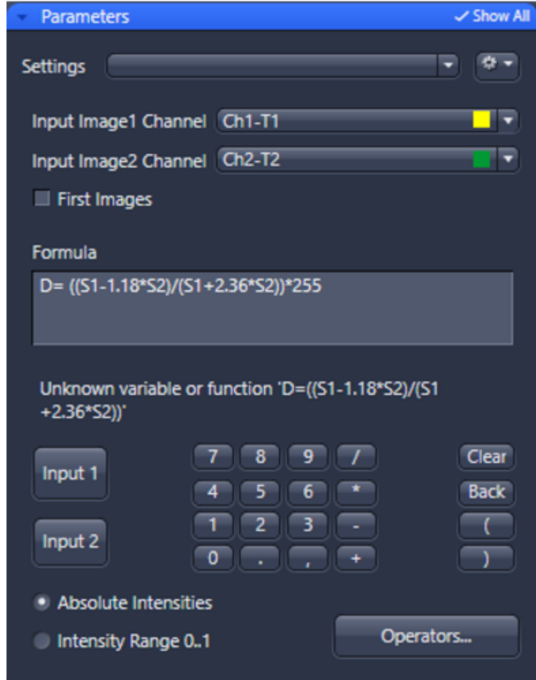
1. Set up a multitrack configuration with alternating analyzers: **P** in Track 1, **S** in Track 2, leaving all other imaging settings as when obtaining the image series for determining the G factor.
2. Acquire a time series.
3. In MeanROI View choose the **Ratio Type 3 formula** in the **Ratio** tab.



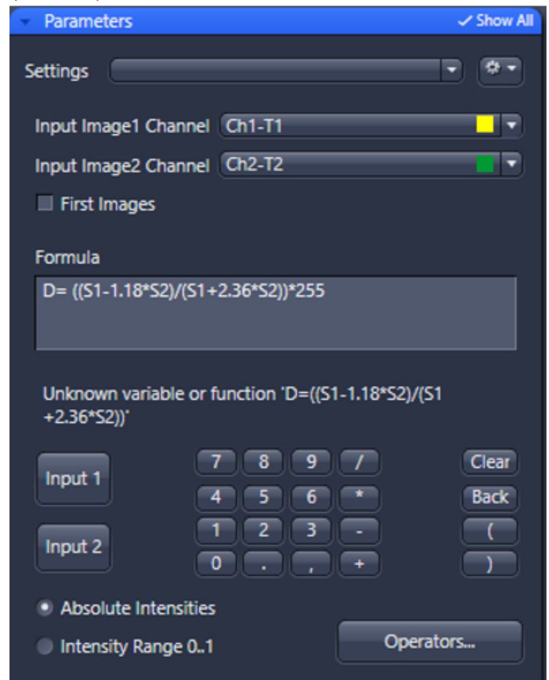
4. Set **Background Correction** to **Constant**.
5. Type in the value of the G Factor (as “Background Correction) as a multiplier into the nominator and 2x G Factor as (as a “Background” Correction) into the denominator. With Ch1-T1 and Ch1-T2 corresponding to IP and Is this formula corresponds to the formula for anisotropy r.
6. Adjust multiplier and add value as fit to get a good image representation.

7. Alternatively, open **Processing | Method | Image calculator**.





8. Use source 1 (S1) being the P track image (Ch1 T1) and source 2 (S2) the S track image (Ch1 T2) with consideration of the G factor in **Parameters**.



14.1.2 FCS (Fluorescence Correlation Spectroscopy)

FCS (FCS) records temporal changes in the fluorescence emission intensity caused by single fluorophores passing the detection volume. It provides quantitative localized measurements of physical parameters including mechanisms of transport, molecular mobility, and densities of fluorescently labeled molecules based on temporal fluctuations which are detected via their emission signal intensity. In the most basic configuration, FCS examines the inherent correlations exhibited by the fluctuating fluorescent signal from labeled molecules as they transit into and out of a specified excitation volume. The most easily observed change in intensity is the fluctuation of the concentration of fluorescent molecules. The temporal auto-correlation of the recorded signal intensity provides a quantitative measurement of the strength and duration of the intensity changes. These parameters are used to calculate the average number of molecules and their average diffusion time through the excitation volume. From this, further parameters can be deduced like concentration and size (shape) of the molecule. The dual-color variation, termed Fluorescence Cross-Correlation Spectroscopy (FCCS), is utilized to probe two species labeled with different fluorophores. FCCS can extend investigations to the examination of biochemical reactions between two partners, such as reaction rates, kinetics, fractions of binding or reacting molecules, and mobilities of a complex formed between the partners.

For successful FCS measurements both, the used hardware and the sample must meet certain standards. FCS-based-techniques measure fluctuations caused by single molecules within a small volume of typically less than a femtoliter which needs to be precisely defined. Hence a high-end objective paired with highly sensitive detectors and a low noise highly stable laser source is a prerequisite. Especially for FCCS measurements color shift effects of optical elements have a negative impact onto the results. Hence one must keep the measurement spot on the optical axis/center of the scan field when FCCS data are acquired. The stage rather than the scanner need to be used to position the measurement spot in the very center of the scan field.

FCS measurements need a single photon counting detector and a laser wavelength suitable for fluorophore excitation. FCCS measurements are typically performed observing two fluorophores excited with different wavelengths and using two counting detectors.

LSM 980 provides several detectors which can be operated in counting mode and hence can be used for FCS and FCCS measurements.

The most basic configuration includes two counting capable channels, more advanced systems provide 5 or even 7 counting capable channels. In addition, BiG.2, an external detection unit which can be mounted onto the scan head of each of these configurations, can be used for FCS and FCCS.

FCS and FCCS measurements are either performed using dissolved molecules/dyes or using cultured cells observing low to medium abundant proteins (few pM up to µM) at endogenous expression levels.

For successful measurements the preparation of the sample must meet some prerequisites.

When analyzing labeled molecules in solution the labeled molecule/dye should be dissolved in a water/buffer solution and diluted such that the concentration is between 1-50 nM. After dilution, the solution must be put into a chamber with a # 1.5 cover-glass bottom. The glass bottom thickness/quality is influencing the measurement which relies on a precisely defined focal spot. Make sure to put enough media to cover the entire bottom of the chamber. Common dyes used for this kind of experiment are for example any kind of Alexa Fluor® dye.

For cultured cells the endogenous expression of fluorescently labeled proteins is best achieved using modern genome editing techniques. This keeps the expression level low enough for successful FCS and FCCS measurements.

14.1.2.1 Overview Acquisition Tools

Imaging Setup

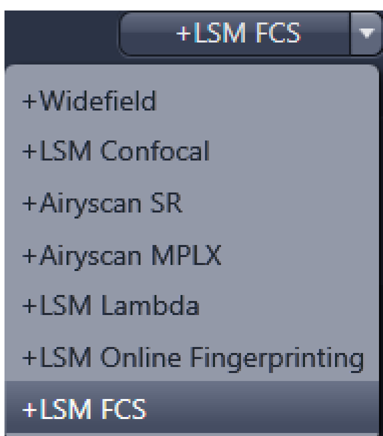


Fig. 87: Imaging Setup tool, FCS track selection

Within the Imaging Setup tool select **+LSM FCS** from the track selection drop down list to set up and activate a FCS track. Only with an active FCS track, the FCS specific acquisition tools will be displayed.

Main Action Buttons

Parameter	Description
Continuous	Opens a new FCS document and starts a continuos FCS measurement according to the settings defined in the **Channels Tool**.

Parameter	Description
Snap	Starts the FCS measurement according to the settings defined in **Channels Tool** and **Experiment Regions**. Snap performs a FCS data acquisition at the center of the scan field or at all FCS spots defined and activated in **Experiment Regions**.

Channels Tool

For description of the parameters in the **Channels** tool, see *Channels Tool - Measurement Settings [▸ 920]*.

Right Tool Area

Parameter	Description
Detector Counting Tool	Shows counts per second (Count), correlation amplitudes (Correlate) or counts per molecule (CPM). The values are updated whenever a FCS acquisition is running. The values displayed can be averaged over 1, 3 or 10 seconds.

Experiment Region Tool

Parameter	Description
Draw FCS spot	Defines positions in xy (FCS spots) for FCS measurement using the scanner for positioning.
Update Z	Allows to assign different Z positions to different FCS spots. Z-Focus positions are only indicated and relevant for FCS spots, not for any other experiment regions..

File Menu

Parameter	Description
New FCS Document	Opens a new FCS document in the Center Screen Area. Existing data can be added to the new document by copy and paste or drag and drop from stored documents to rearrange and combine data sets.

FCS Document View

Parameter	Description
Correlation view	Opens when data acquisition starts. It shows count rate and correlation graphs for all active acquisition channels.
Info	Shows selected information on the experiment.
Fit	Provides various tools for data fitting and display.

14.1.2.1.1 Channels Tool - Measurement Settings

Parameter	Description
Measurement Time	Here you can enter the period of one measurement.
Repetitions	Here you can enter the number of repetitions for one measurement.
Adjust	Opens the Adjust Pinhole wizard to perform an automatic count rate versus pinhole position scan.
Z-scan	Opens the *Z-Scan [921]* wizard to perform a Z-scan ant plot the count rate averaged value dependent on the Z position to find the optimal focus position.
Fit Model	Here you can select a fit model for fitting the data of the selected channel after finishing the measurement. A different fit model can be selected for each FCS/FCCS channel.

14.1.2.1.1.1 Adjust Pinhole Wizard

Info

In case two lasers and two detectors (for the visible light path) are defined, the plot shows more curves, one for each laser/detector pair. Potential cross-talk effects can be excluded when defining only one laser and detector for the adjustment procedure. The curves match the color of the detector defined in the **Imaging Setup** or **Channels** tool.

Parameter	Description
Invisible light path	Only displayed if the laser with invisible light (405 nm or IR laser) is defined for the acquisition.
– Adjust X axis	Adjust the position of the pinhole on the X axis.
– Adjust Y axis	Adjust the position of the pinhole on the Y axis .
– Coarse	Moves the pinhole position in X or Y over the whole range while acquiring FCS data for each position. The count rate data are averaged, fitted to match a gaussian curve and plotted against the pinhole position. The plot is displayed in the center area in the **Calibration View** tab. It is possible to change between the view tabs during acquisition.
– Fine	Moves the pinhole position in X or Y about 10 micrometer around the currently stored value while acquiring FCS data for each position. The count rate data are averaged, fitted to match a gaussian curve and plotted against the pinhole position. The plot is displayed in the center area in the **Calibration View** tab. It is possible to change between the view tabs during acquisition.

Parameter	Description
– Pinhole position sliders/ input box	Before the adjustment scan is started, the slider and the input box beneath the show the currently stored pinhole position in X and Y. The sliders can be moved and the position edited once the scan has finished. This allows to move the positions to a different value than suggested by the system. The then indicated value will be stored when clicking **Finish** to leave the wizard. The Pinhole position in the plot is shown in micrometer. One micrometer corresponds to approximately one motor step.
Visible light path	Only displayed if the laser with visible light ((lasers from 445 nm to 639 nm) is defined for the acquisition.
Cancel	Cancels the procedure and keeps the existing pinhole position.
Finish	Takes over the new pinhole position as general position also for all other LSM acquisition functions.

14.1.2.1.1.2 Z-Scan Wizard

Z-Scan provides an automated procedure to optimize focus position for auto- or cross correlation measurements. The **Z-Scan** provides the following parameters:

Parameter	Description
Current Position	Displays the current position. This position is changing during the actual scan procedure.
Center Position	Displays the Center Z Position of the defined range.
Range	Define the range of the Z-Scan by either typing in a value or using the scroll arrows of the Range edit box.
Step Width	Define the step width by either typing a value or using the scroll arrows of the Step Width edit box.
Start	Perform the scan by pressing Start. During the scan the count rate is plotted for the selected channels using the channel assigned colors.
	Once the scan is complete a red bar is positioned at the Z position with the maximum count rate. This position can be changed by moving the red bar, moving the slider beneath the Start button, typing in a value into the edit box next to the slider, or using the scroll arrows.
Finish	Click Finish to take over this Z position for the FCS measurement.
Cancel	Click Cancel to discard the values and keep the original Z position

14.1.2.2 FCS Document View

This view contains three layers, which can be selected by clicking the corresponding tab:

Tab	Description
Correlation	Displays the count rate trace, the correlation function, the photon counting histogram, the pulse density histogram, and the result table, see *Correlation Tab [▶ 922]*.

Tab	Description
Fit	Displays the fit graph, the residuals, and the used model, see *Fit Tab [▸ 927]*.
Info	Displays the defined name, any typed in comment, and some of the meta data, see *Info Tab [▸ 932]*.

If you close an FCS document without saving, you will be asked in the **Close image** dialog, if you want to save the data.

14.1.2.2.1 Correlation Tab

Fig. 88: Correlation tab

1	*Count rate diagram [▸ 923]*
2	*Pulse distance histogram [▸ 923]*
3	*Correlation diagram [▸ 924]*
4	*Photon counting histogram (Pulse density histogram) [▸ 925]*
5	*Result table [▸ 926]*
5	*FCS view options [▸ 933]*

14.1.2.2.1.1 Count Rate Diagram

In the **Count Rate diagram** the count rate(s) (CR) in kHz is plotted vs. running time. If a cross correlation set-up is used, the count rate trace for each channel is displayed.

Zoom into the diagram by pressing the left mouse button and drawing a rectangle of the area you want to zoom in. After disengaging the button, the zoom image is displayed.

Clicking the right mouse button within the diagram opens the **Count rate** context menu.

Parameter	Description
Reset diagram zoom	Resets to the original image size when zoomed in.
New cut region	When raw data files are available, define cut regions by choosing the **New cut region** option. The cut region size can be adapted by two sliders. The cut out region is thereby displayed as a matted box. Select independent cut regions for different channels of a cross correlation experiments. In cross-correlation calculations, a cut region in one channel will automatically define the same cut region in the other channel. You can select more cut regions by repeatedly choosing this option. These regions may overlap.
Remove cut region	Removes the last cut region. By repeatedly choosing this option, the cut regions in the reverse order of their creation will be removed.
Remove all cut regions	Removes all cut regions simultaneously. The remaining parts of the count rate disrupted by cut out regions will be separately correlated and the average will be calculated and displayed.
Copy text to clip board	Copies the diagram coordinates into the clipboard, from which they can be pasted into other programs like Excel.
Write text to file	Stores the diagram coordinates in a .txt file. You are prompted to choose a name and a folder before saving.
Copy graphics to clip board	Generates an image of the graph with the legend text and color coded graphs which can copied to standard windows documents.
Abscissa logarithmic	Displays the abscissa in a logarithmic scaling.
Ordinate logarithmic	Displays ordinate in a logarithmic scaling.
Line width	Allows to select the thickness of the graph. Six settings are possible.

Tab. 1: Correlation view - Count Rate context menu

14.1.2.2.1.2 Pulse Distance Histogram

This diagram shows the frequency plotted against the times elapsed between two subsequent pulses (or: photons recorded from the detector). To obtain this histogram, the time distance between two photons is evaluated using the raw data.

Zoom into the diagram by pressing the left mouse button and drawing a rectangle of the area you want to zoom in. After disengaging the button, the zoomed area of the graph is displayed.

Clicking the right mouse button opens the **Pulse Distance Histogram** context menu:

Parameter	Description
Reset diagram zoom	Resets to the original image size when zoomed in.
Copy text to clip board	Copies the diagram coordinates into the clipboard, from which they can be pasted into other programs like Excel.
Write text to file	Stores the diagram coordinates in a .txt file. You are prompted to choose a name and a folder before saving.
Copy graphics to clip board	Generates an image of the graph with the legend text and color coded graphs which can copied to standard windows documents.
Abscissa logarith-mic	Displays the abscissa in a logarithmic scaling.
Ordinate logarith-mic	Displays ordinate in a logarithmic scaling.
Line width	Allows to select the thickness of the graph. Six settings are possible.

Tab. 2: Correlation view - Pulse Distance Histogram context menu

14.1.2.2.1.3 Correlation G(τ) Diagram

The diagram shows the correlation functions for each activated channel.

Zoom into the diagram by pressing the left mouse button and drawing a rectangle of the area you want to zoom in. After disengaging the button, the zoomed area of the graph is displayed.

Clicking the right mouse button within the diagram opens the**Correlation** context menu:

Parameter	Description
Reset diagram zoom	Resets to the original image size when zoomed in.
Copy text to clip board	Copies the diagram coordinates into the clipboard, from which they can be pasted into other programs like Excel.
Write text to file	Stores the diagram coordinates in a .txt file. You are prompted to choose a name and a folder before saving.
Copy graphics to clip board	Generates an image of the graph with the legend text and color coded graphs which can copied to standard windows documents.
Abscissa logarith-mic	Displays the abscissa in a logarithmic scaling.
Ordinate logarith-mic	Displays ordinate in a logarithmic scaling.
Line width	Allows to select the thickness of the graph. Six settings are possible.

Parameter	Description
Normalize	Is used to normalize the selected G(0) value corresponding to the number of diffusing particles to 2, not the total correlation to which also other processes like triplet state can contribute. All other values (subtracted by 1) will than be multiplied by the correction factor defined by the ratio of 1 to [G(0)-1] and increased by 1, hence $G(\tau)_{normalized}=[1/(G(0)-1) * (G(\tau)-1)]+1$. Deactivating Normalize will display the normal correlation curve. Normalization only works for fitted data as only then a G(0) value is available.

Tab. 3: Correlation view - Correlation context menu

14.1.2.2.1.4 Photon Counting Histogram

This diagram (also called Photon Counting Histogram) shows the frequency plotted against the photon number in a certain time bin. To obtain this histogram, the number of pulses (or: photons recorded from the detector) in a moving time window are recorded and included in a histogram. Determine the binning when loading a *.fcs file with raw data saved along with it using the **Reload** button.

Zoom into the diagram by clicking+holding the left mouse button and drawing a rectangle of the area you want to zoom in. If the button is disengaged, the zoomed area of the graph is displayed.

Clicking the right mouse button opens the **Photon Counting Histogram** context menu:

Parameter	Description
Reset diagram zoom	Resets to the original image size when zoomed in.
Copy text to clip board	Copies the diagram coordinates into the clipboard, from which they can be pasted into other programs like Excel.
Write text to file	Stores the diagram coordinates in a .txt file. You are prompted to choose a name and a folder before saving.
Copy graphics to clip board	Generates an image of the graph with the legend text and color coded graphs which can copied to standard windows documents.
Abscissa logarithmic	Displays the abscissa in a logarithmic scaling.
Ordinate logarithmic	Displays ordinate in a logarithmic scaling.
Line width	Allows to select the thickness of the graph. Six settings are possible.

Tab. 4: Correlation view - Photon Counting histogram context menu

14.1.2.2.1.5 Result Table

The **Result Table** below the diagrams displays the measuring results. The width of the columns can be changed by moving the border lines. The order of the columns can be changed. For this purpose, click on the head line of the relevant column, hold down the mouse button and move the column to the required position. When the mouse button is released, the column is inserted in the new position. A scrollbar at the bottom of the table allows one to view all parameters that might not fit within the width of the table. A scrollbar on the right allows access to all repetitions.

1. Select a line in the table by clicking on it with the mouse (multiple choice is possible by pressing the **Shift** or **Ctrl** key additionally).
   → Selected lines are highlighted in color and displayed in the legends of the diagrams.
   → Corresponding graphs are shown color coded in the graphic displays.
2. Select lines and define properties of the table by pressing the right mouse button, when the cursor is within the table.
   → The **Result Table** context menu opens offering different options.

Parameter	Description
Select all	Selects all measurements (rows) in the table regardless which line is highlighted.
Select all channels	Selects all rows belonging to the same channel of a repetitive measurement as the highlighted row.
Select all repetitions	Selects all rows belonging to the same repetitive measurement as the highlighted row.
Select all positions	Selects all rows belonging to the same measurement position as the highlighted row.
Select all kinetic indices	Selects all rows belonging to the same kinetic time point as the highlighted row.
Delete	Deletes the highlighted rows.
Cut	Stores in the clipboard the highlighted rows. Only if the data are pasted in a new window, the data are deleted from the old one.
Paste	Pastes rows currently stored in the clipboard into the table.
Color	Allows to select a color for the selected data sets.
Copy text to clipboard	Copies the table contents into the clipboard, from which they can be pasted into other text programs.
Write text to file	Stores the diagram coordinates in a .txt file. You are prompted to choose a name and a folder before saving.
Properties	Opens the **Table Properties** window.

Tab. 5: Result Table context menu

14.1.2.2.2 Fit Tab

The **Fit** tab provides access to the tools which allow to work with newly generated or already existing data for data analysis.

Fig. 89: FCS Document, Fit tab

1	*Diagram area [▸ 927]*
2	*Channel area [▸ 929]*
3	*Result table [▸ 932]*

14.1.2.2.2.1 Diagrams

Depending on the chosen Fit model, the diagrams differ.

When fitting to a correlation model the upper graph shows the **Correlation G(τ) Fit diagram** (correlation plotted versus the correlation time) with the overlaid fit graph. It also displays the fit range defined by the red (start value) and the blue (end value) bars. The lower **Fit deviation** curve depicts the fit residuals. If an experiment is loaded or data have just been acquired for which no fit has been conducted yet, only the measured **Correlation G(τ)** curve is shown and no overlaid fit curve. The **Correlation-Fit deviation** diagram is still empty.

For fitting to a PCH model the upper graph shows the **Frequency diagram** (frequency against count rate per bin) with the overlaid fit graph. If an experiment is loaded for which no fit has been conducted yet or data have just been acquired , only the measured curve is shown and no fit curve. The **Frequency-Fit** diagram will be empty.

Zoom into the diagrams by pressing the left mouse button and drawing a rectangle of the area you want to zoom in. If the button is disengaged, the zoomed area is displayed.

Clicking the right mouse button within the diagrams opens a context menu each for the Correlation, the PCH fit diagram, and the Fit deviation diagram.

Parameter	Description
Reset diagram zoom	Resets any zoomed image
Copy text to clip board	Copies the diagram coordinates into the clipboard, from which they can be pasted into other programs like Excel
Write text to file	Stores the diagram coordinates in a .txt file. You will be prompted to choose a name and a folder before saving.
Copy graphics to clip board	Generates an image of the graph with the legend text and color coded graphs which can be copied to standard windows documents
Abscissa logarithmic	Displays the abscissa in a logarithmic scaling
Ordinate logarithmic	Displays the ordinate in a logarithmic scaling When ordinate is set to a logarithmic scale all zero frequencies will be displayed as vertical tabs, since the log0 goes to infinity low values.
Line width	Allows to select the thickness of the graph. Six settings are possible.
View measured data	Displays the measured Correlation or PCH curve in addition to the Fit curve. If this option is not selected, only the Fit curve is displayed.
Normalize	Is used to normalize the selected G(0) value corresponding to the number of diffusing particles to 2, not the total correlation to which also other processes like triplet state can contribute. All other values (subtracted by 1) will than be multiplied by the correction factor defined by the ratio of 1 to [G(τ)-1] and increased by 1, hence $G(\tau)_{normalized}$=[1/ (G(τ)-1) * (G(τ)-1)]+1. Normalization only works for fitted data as only then a G(0) value is available. This option is only available for the Fit Correlation diagram.
Show fit range text	Displays the start and end values of the fitted data of the correlation curve defined by the red and blue bars. The positions of the bars can be adjusted by drag and drop. Start Channel and End Channel determine which part of the correlation curve should be fitted to the model. The start and end position of the channels are displayed as correlation times (in µs). This option and the bars are only available for the Fit Correlation diagram.
Scaling	Opens the Diagram scaling window . Enter the required percentage value (from 1 to 100) for the scaling and click on OK to rescale, Cancel to keep the old value. The scaling of the G(t) axis is adjusted. This option is only available for the Fit deviation diagrams.

Tab. 6: Diagram context menu

14.1.2.2.2.2 Channel Area

The **Channel** area within the **Fit View** tab shows the active channel (name and color), for which the **Fit** and **Fit All** buttons apply. If more than one row is selected (shown in blue color in the result table) the active channel is the channel in the last selected row of one or multiple selected channels.

- **Fit** applies to all selected rows of all channels.
- **Fit All** applies only to the rows belonging to the active channel.

The **Channel** area provides the interface to define and save a Fit model. This interface can only be accessed when either:

- a continuous FCS acquisition has been started
- an FCS experiment has been performed
- a new FCS document has been opened and data have been dragged into this document.

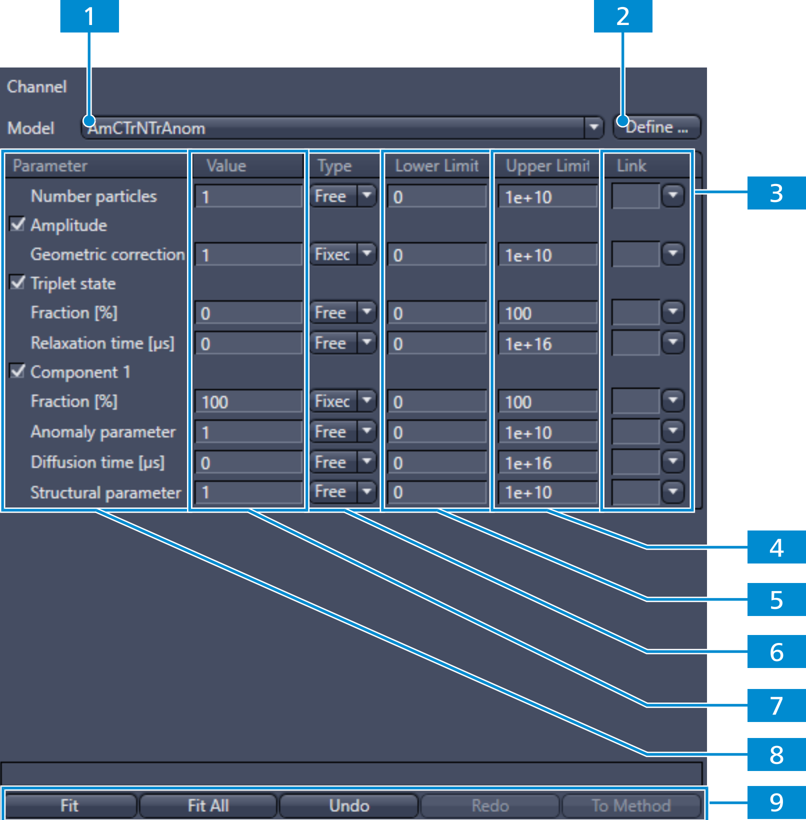


Fig. 90: Fit tab, Channel area

1	**Model** drop down list
2	**Define...** button
3	**Link** edit box with drop down list
4	**Upper Limit** display box
5	**Lower Limit** display box
5	**Type** edit box with drop down list
6	**Value** edit box
7	**Parameters** list

8 *Fit action buttons [▸ 931]*

Parameter	Description
Model	Drop down list contains all previously defined and stored fit models. Selecting a model by clicking onto the name in the list loads the model and displays a set of parameters which can be individually adapted when applying the fit model to the data set(s). The parameters are updated once the fit model is applied to the data.
Define	Click to define a new Fit model. For a detailed description of the available parameters, see *Define Model Dialog [▸ 939]*.
Link	Links the parameter globally. Type the required letters, separated by comma. ▪ **M**: links the parameter over different measurements ▪ **K**: links the parameter over kinetic indices with the same time points ▪ **P**: links the parameter over positions obtained from measurements at the same site ▪ **R**: links all repetitions of one measurement ▪ **C**: links the parameter for the same channel Alternatively, select the link(s) from the drop down list opened when clicking onto the drop down arrow. If a linkage is activated that does not apply for a measurement, it is disregarded. Otherwise the same rules are in place as for **Fit** and **Fit all** in terms of to which data rows the links apply.
Upper Limit	Defines the upper limit value tolerated from a fit; if this value is exceeded, the fit is rejected and another (global) maximum is searched for. The default value depends on the parameter. The **Upper Limit** parameter value is only accessable if the **Show Limits...** option is selected within the interactive selection list opened with a right mouse click into the parameter panel.
Lower Limit	Defines the lower limit value as a fit parameter; if this value falls short, the fit is rejected and another (global) minimum is searched for. Change the value by editing the default value in the edit box. The default value depends on the parameter, but is in most cases 0. The **Lower Limit** parameter value is only accessable if the **Show Limits...** option is selected within the interactive selection list opened with a right mouse click into the parameter panel.
Type	Displays whether a parameter is free floating, if its value is fixed or whether it should be used as a start value. Choose between the options in the drop down menu by selecting **Free**, **Start** or **Fixed**, respectively. ▪ When **Free** is selected, the parameter will be defined as free. After the fit, the field will contain the fit result for the parameter. The start values of the parameter (initial guesses) will be calculated by an algorithm. For user defined models the option **free** is not available as a setting for the parameter **Type**.

Parameter	Description
	▪ **Fix** sets a fixed parameter which is useful if the value is known from other measurements. ▪ **Start** assigns a start value to the parameter and leaves the parameter free to fit. In this case, no initial guesses will be made.
Value	Displays the currently assigned value of the parameter. Change the value by editing the default value in the edit box..
Parameter	Displays the parameter's name. Activate or deactivate the parameter by ticking / de-ticking the check box. Only if a parameter is active it will be taken into account for fitting the data.

- The parameter settings are not stored with the experiment or the fit model. They need to be defined anew for each fit procedure.
- A text in the state display panel warns in yellow writing on any constraints or errors of the defined fit model and suggests suitable changes. For example, whenever some inconsistencies are present, e.g having two parameters free that depend on each other, like the geometric factor and the number of molecules, the system gives a warning about the mistake.

Info

Generally it is accepted that non-linear fitting procedures yield more reliable results when the number of free floating parameters is low. It is recommended to fix parameters which are known from independent measurements. Good candidates for fixing are diffusion times of the free dye and the free (i.e. not bound) partner, which can be determined in previous measurements. Another good candidate is the structural parameter that is an instrumental parameter.

The quality of the fit is displayed in the **chi**^{2} display box of the **Fit table**. The χ^2 (chi^2) value should approach zero for highest quality. The range of the data to be used for fitting can be defined by re-positioning the red and blue bar originally set at the beginning and end of the correlation diagram to the required start and end range positions. For the then next Fit procedure then new range is applied.

Lower and upper limits are only displayed if selected. To select, click with the right mouse button into the result table to open the context menu.

14.1.2.2.2.2.1 Fit Action Buttons

Parameter	Description
Fit	Performs a fit for all highlighted measurements (rows) according to the loaded model. When the fit is completed, the free parameters are replaced with the new fitting results and the fit graph and result table are updated.
Fit all	Performs a fit for all measurements (rows) that have the same channel as the one displayed in the **Channel** display field (active channel). All other channels are ignored. Changing to a row with a different channel loads the last assigned model for that row.
Undo	Cancels a previous command. Repeatedly pressing will cancel commands in the reverse of their execution.

Parameter	Description
Redo	Re-executes a previously undone command and hence will become only active, if an action was performed. Repeatedly pressing will re-execute previous undone commands in the order of their previous execution.
To Method	This function is not supported.

14.1.2.2.2.3 Result Table

The data result table in the **Fit** view displays the values of the fitted parameters. It is updated when clicking the **Fit** button in the **Model** panel. Measuring rows in the **Result** table can be activated / deactivated by checking / unchecking the corresponding check boxes. Deactivation of these check boxes will not exclude the relevant rows from all subsequent evaluation procedures, but only from the average. Average curves are updated in respect to the rows taken into consideration.

14.1.2.2.3 Info Tab

Info

The complete experiment settings, including all multidimensional acquisition settings, are stored with the experiment when it is saved.

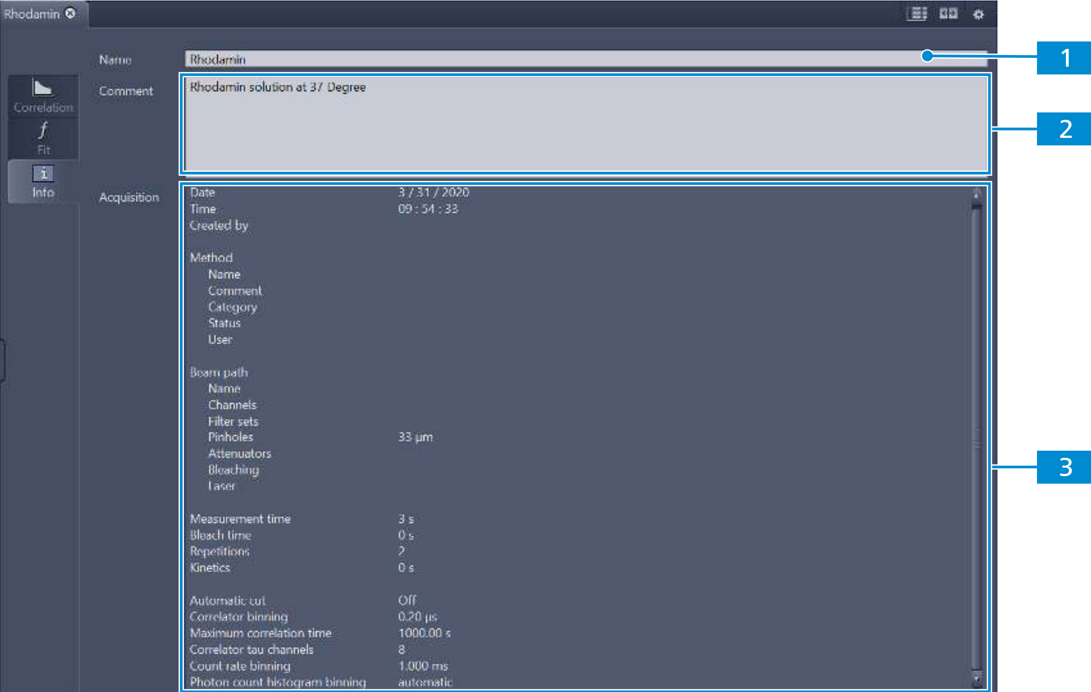


Fig. 91: Info tab

1 **Name** display box

The name of the experiment is updated whenever the experiment is saved under a defined name. It cannot be edited otherwise.

2 **Comment** edit box

Write down any relevant information about the experiment you would like to keep with the data.

3 **Acquisition** display box

The panel shows apart from the acquisition date and the pinhole size only information about the single measurement parameters.

See also

Fit Action Buttons [▸ 931]

14.1.2.2.4 FCS Document View Options

14.1.2.2.4.1 Correlation Tab

The **Correlation** action tab lists all correlation channels with their assigned channel color including cross correlation channels.

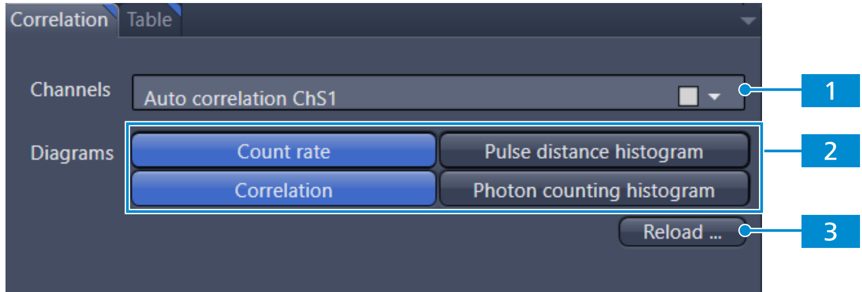


Fig. 92: FCS Document, Correlation action tab

1 **Channels** dropdown menu

Displays the list of active channels and allows to change the color when clicking on the arrow. Changes are immediately effective.

2 **Diagrams**

Choose the diagrams to be displayed by activating the corresponding buttons. Selected diagrams will be highlighted in blue. By clicking on the appropriate button, the diagram can be toggled between **ON** and **OFF**.

3 **Reload ...** button

Opens the **Reload** tool (see *Reload Tool [▸ 935]*).

14.1.2.2.4.2 Table Tab

The **Table** action tab provides several options to sort and display the data.

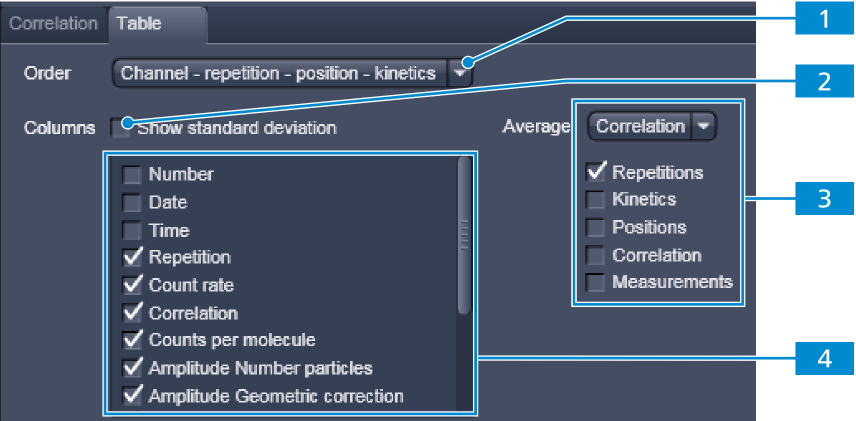


Fig. 93: FCS document, Table action tab

1 **Order** drop down list

Determine, how different measurements are grouped together. The hierarchy of grouping of categories is determined by their listed sequence.

2 **Show standard deviation** checkbox

If activated, all parameters are displayed with their values and their calculated standard deviation from the different measurements. Only then the displayed average correlation function can be meaningfully fitted.

3 **Average** drop down list with correlation measurements

In the drop down selection choose how to average the data.

- When **Fit results** is selected, the single values of the parameters are averaged.
- Selecting **Correlation** averages single data points of each correlation function and an average correlation function is calculated.
  Check, which measurements (Repetition, Kinetics, Positions, Correlation) should be used to built the average by activating the corresponding checkboxes.

4 **Parameters** list

Only parameters set active are visible in the table. The order of the displayed parameters can be adjusted using drag and drop in the table itself. Deactivation of these check boxes excludes the relevant rows from all subsequent evaluation procedures like averaging. These settings will be stored when saving the data. Immediate reactivation is possible when activating the check box. The scroll bar allows viewing all content of the display box.

14.1.2.2.4.3 Reload Tool

Whenever a *.FCS file is opened and raw FCS data (*.RAW files) have been saved during acquisition along with the *.FCS file, the **Reload ...** button is available in the **Correlation** tab in the FCS document view.

The tool allows to redefine several parameters for

- *Correlation [▸ 935]*
- *Count rate [▸ 936]*
- *PCH (Photon Counting Histogram) [▸ 937]*
- *Electronic dust filter [▸ 938]*

The panel opens with the default settings which are applied for the original data acquisition.

14.1.2.2.4.3.1 Correlation

The **Correlation** tab allows to specify how the raw data will be processed for correlation analysis.

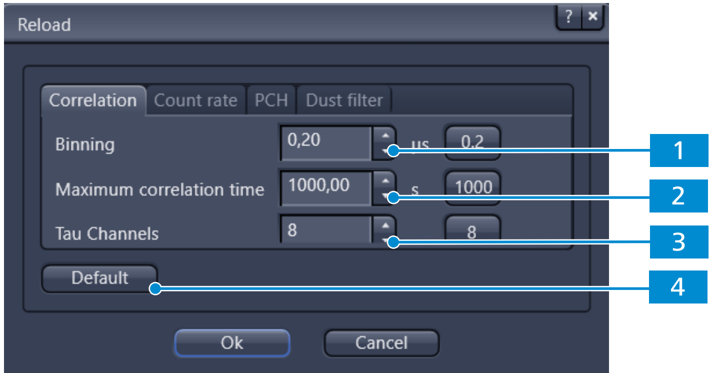


Fig. 94: Reload tool, Correlation tab

1 **Binning time** edit box

Determines the minimal binning time used in the correlation analysis and also the start correlation time, which will be identical to minimal binning time. Chose whether to directly enter values or use the scroll arrows. Can be reset to the default value by clicking the **0.2** button.

2 **Maximum correlation time** edit box

Defines the end correlation time. It cannot exceed the measurement time. Hence, the value will only be used if equal to or smaller than 2/3 of the measurement time, else the measurement time will be used automatically. Chose whether to directly enter values or use the scroll arrows. Can be reset to the default value by clicking the **1000** button.

3 **Tau Channels** edit box

Defines the number of linear increased Tau channels per multiple Tau step. Chose whether to directly enter values or use the scroll arrows. Can be reset to the default value by clicking the **8** button.

4 **Default** button

Resets all parameters to the default values. Please note that with the default settings, the algorithm works the fastest.

The algorithm for auto-correlation functions is as follows:

In the first correlation step all events within a certain correlation interval T are counted.
The default T in the first correlation step (n=0) equals 200 ns, which is 4x the maximum sampling rate of 50 ns, as limited by the dead time of the detectors and the electronics. T will be changed by the entry in the **Binning** display window. This corresponds to a clock speed of 20 MHz.
Thus, for this correlation interval a maximum of 4 events can be counted. These events / correlation intervals are shifted against themselves for a time interval t. The first t equals the first T value, as a default 200 ns = 0.2 ms. t will be changed by the entry in the **Binning** display window.
T is linearly increased by step sizes of 200 ns (or the entered value) for 8 times (direct tau) and than for another 8 times (multiple Tau), i.e. the maximum correlation time in the first step is 16 x 0.2 ms = 3.2 ms using the default settings. The direct and shifted events / correlation intervals are multiplied interval for interval and the products are added together and normalized.
The normalization result is defined as: (result x received clocks)/(pulse2) for auto-correlation or (result x received clocks)/(pulseA x pulseB) for cross-correlation. The received clocks counted equal 4x the number of correlation intervals, which are defined by the measurement time. At the end the relevant data for the next correlation step are processed. Here, the correlation interval is increased by a factor of 2. Hence, the correlation interval (T) in the n-th correlation step is T = 0.2 ms x 2n for the default settings. The initial lag time for a certain correlation step corresponds to the last lag time of the previous correlation step added to the respective correlation interval. This value is then linearly increased 8 times.
Therefore after step n, the correlation or lag time (t) ultimately corresponds to t = 6 x 0.20 + 8 x 0.2 x 2n ms. The doubling of the correlation interval is performed on default 25 times. Thus the maximum is: t = 16 x 0.2 + 8 x 0.2 x 225 ms.

14.1.2.2.4.3.2 Count rate

The **Count rate** tab allows to specify the binning time used for the count rate trace.

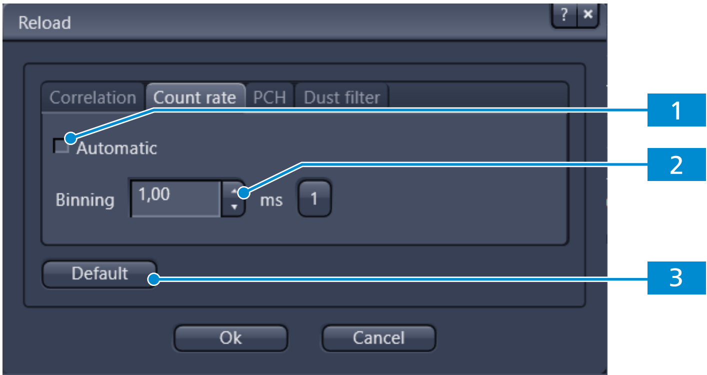


Fig. 95: Reload tool, Count rate tab

1 **Automatic** checkbox
Activates dynamic binning.

2 **Binning time** edit box
Determines the constant binning time used in the count rate analysis. Chose whether to directly enter values or use the scroll arrows. Can be reset to the default value by clicking the **1** button.

3 **Default** button
Resets all parameters to the default values. Please note that with the default settings, the algorithm works the fastest.

When **Automatic** is active, the system averages three data points and rebinds the data in dependence of the measurement time. The diagram will have a mean with fluctuations above and below the mean.
In constant binning, data points are not averaged. This will result in a baseline with fluctuations above.

In (automatic) dynamic binning the count rate trace will be adjusted to the measurement length. The count rate trace represents the averaged binned count rate versus measurement time, in other words photons/second therefore intensity.
The bin window in dynamic binning now depends on the measurement time, whereby the bin window doubles, if 500 values are exceeded.
In the first step, the binning time is 3.2 ms. For the next steps, the binning time (t_r) becomes t_r = 3.2 ms x 2n. The measurement time (t_d), at which the binning time doubles is calculated according to t_d = 3.2 ms x 500 x 2n.

14.1.2.2.4.3.3 PCH

The **PCH** allows to specify the binning time used for the photon counting histogram.

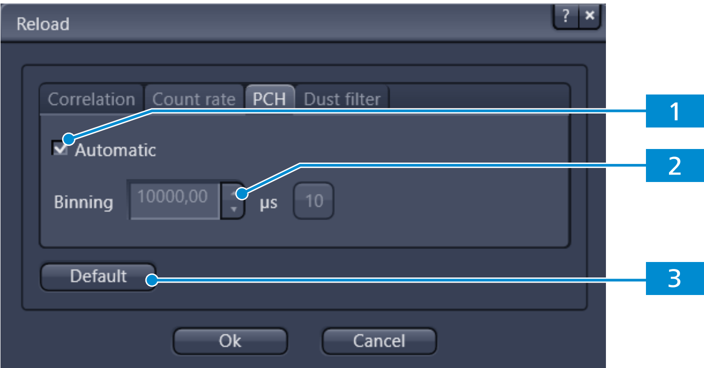


Fig. 96: Reload tool, PCH tab

1 **Automatic** checkbox
Activates dynamic binning.
When **Automatic** is active, 32 different binning times will be used.

2 **Binning time** edit box
Determines the constant binning time used for the PCH. Chose whether to directly enter values or use the scroll arrows. Can be reset to the default value by clicking the **10** button.

3 **Default** button
Resets all parameters to the default values. Please note that with the default settings, the algorithm works the fastest.

Info

In automatic binning mode, binning starts with a value of 50ns, which is doubled 32 times. So binning times are 50 x 2n, with n=1 to 32. The histogram with the best dynamic range (three standard deviations) will be selected and displayed.

14.1.2.2.4.3.4 Dust filter

The **Dust filter** tab allows to activate an electronic dust filter. Define a threshold in the **Count rate** intensity that, if exceeded, will lead to a removal of the corresponding count rate region from the correlation analysis.

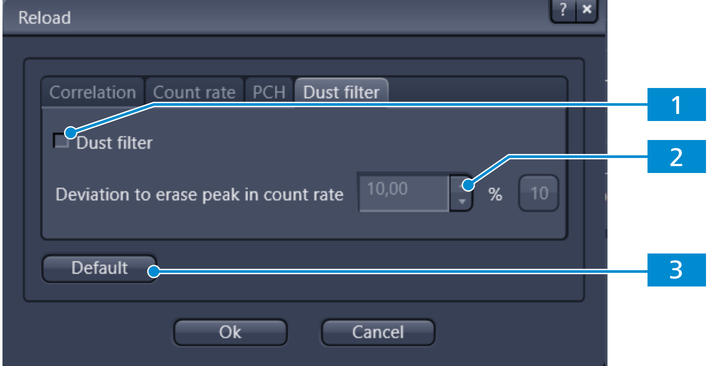


Fig. 97: Reload tool, Dust filter tab

1 **Dust filter** checkbox

Activates the dust filter function.

2 **Deviation to erase peak in count rate** edit box.

Determines the Threshold in %. Chose whether to directly enter values or use the scroll arrows. Can be reset to the default value by clicking the **10** button.
All measurement points within a binned count rate time window having a deviation of more than the specified value from the average count rate will be cut out and not used for the correlation analysis.

3 **Default** button

Deactivates **Dust filter** and resets the values to default.

Note that the cut off count rate is defined as a value exceeding the average count rate during a certain measurement period (bin window) by a certain percentage. Thus, the consecutive fast succession of low peaks might accumulate the same count rate as one high peak within a certain period of time and hence, the cut off is not defined by the peak height but rather by the counts per binning time.

If the integrated count rate over a certain count interval exceeds the average count rate by that threshold, this special interval is discarded for correlation analysis. For example, if the system detects a count rate in a certain time interval that exceeds the average count rate by over 30% and the threshold was set to 30, this interval will be discarded for correlation analysis. The time intervals before and after the discarded region are separately correlated and the results averaged. This holds also true, if more than one region is discarded. In this case all the single regions that are separated by cut out regions are separately correlated and the resulting average is displayed.

Note, that calculation of an average will be performed at the beginning of the measurement. If peak count rates will come at the beginning, this kind of dust filter does not work. Also, due to the necessity to average signals over a certain integration time, more than only the peak area will be discarded. Another outcome of the necessity to average the count rate signal is that several small peaks following close to each other will be treated as a huge peak and might be cut out. This means, in the **Automatic cut** mode accumulated count rates rather than peaks are removed. For cross-correlation experiments, any of the regions discarded in either autocorrelation function will not be used. Cut off regions are framed by stippled boxes and appear matted in the **Count Rate** window.

14.1.2.2.5 Define Model Dialog

Parameter	Description
Correlation	Create a correlation model from predefined equations, which will be fitted analytically, see Generating a Correlation Model.
PCH	Create a photon counting histogram model, which will be fitted numerically, see *PCH tab [▸ 940]*.
Formula	Create a user defined model equation, which can be fitted analytically, see *Formula tab [▸ 941]*.

14.1.2.2.5.1 Correlation tab

This tab show the options for assembling a correlation model. Correlation allows assembling a model with predefined terms.

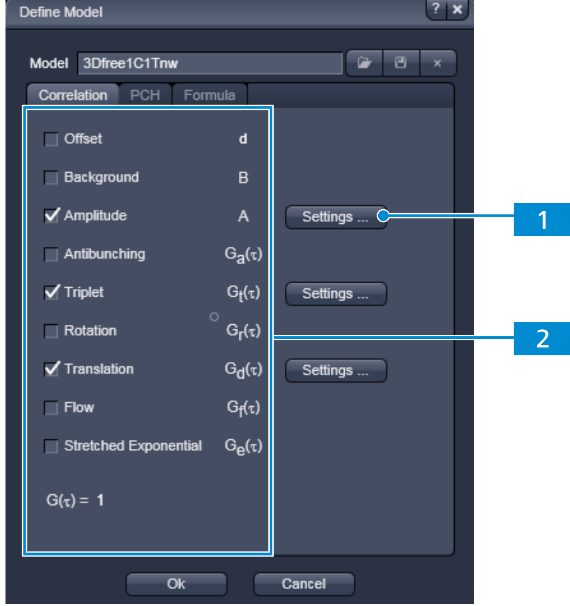


Fig. 98: Define Model dialog, Correlation tab

1 **Settings ...** button

Opens a submenu for the respective term, see *Terms and Term Settings [▸ 952]*.

2 Terms list

Shows selected terms of the currently active model. The assembled equation is displayed in the **G(τ)=1** display area.

A new model can be defined by activating the requested equation terms and defining the corresponding settings for each term.

14.1.2.2.5.2 PCH tab

The **PCH** tab within the **Define Model** tool is used to determine concentrations and the molecular brightness of molecules.

PCH can be fitted only one-dimensional. If a cross-correlation measurement is activated, the PCH model is automatically replaced by a correlation model.

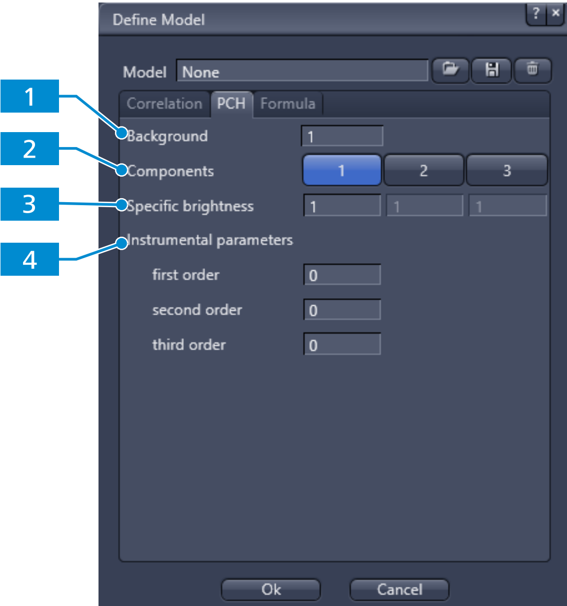


Fig. 99: Define Model dialog, PCH tab

1 Background

- Type in a value if known.
- Keep the value to zero, if no background is expected.
- The background is no fit parameter.

2 Components

Select numbers of components (1, 2 and 3) by clicking the Components **1**, **2** or **3** buttons. The number of active components will be highlighted in blue.

3 Specific brightness

Type in a brightness value (Hz) specific for the chosen number of components beneath the Components button activated. If you don't know the brightness, keep the value at 1.

4 Instrumental parameters

Type in values in the respective **first order**, **second order** and **third order** edit boxes.

These values have to be determined in independent calibration experiments using a defined dye solution with a known brightness. The parameters correct for the deviation of the confocal volume from a true Gaussian distribution. Normally, only a correction for the first order parameter is necessary and recommended. Hence, in the calibration fit keep the first order parameter free and the second and third parameters fixed to zero. Enter the determined first order number and save it with the model for later measurements.

14.1.2.2.5.3 Formula tab

The **Formula** tab allows to define own fit equations.

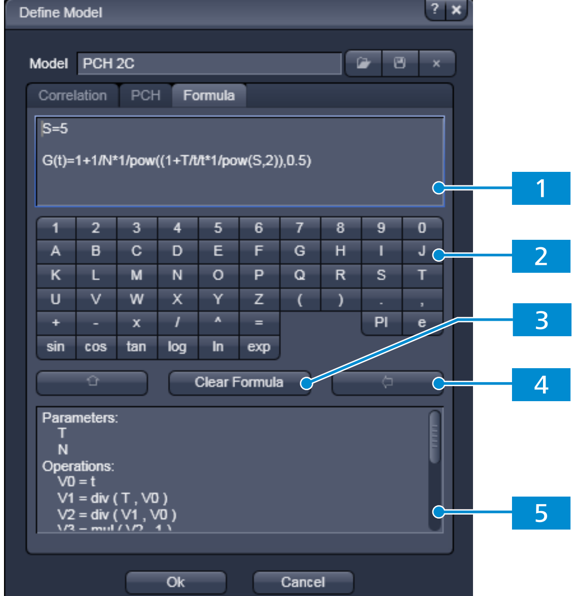


Fig. 100: Define Model dialog, Formula tab

1 **Formula** edit box

Displays the entered formula.

2 **Keyboard**

Used to type the required formula.

3 **Clear Formula** button

Finishes the variable definition process.

4 **Return** button

Deletes the entered formula.

5 **Description** panel

Displays any expected operation and syntax errors.

Note that:

- All variables defined at the beginning are considered fixed variables, all other variables are considered fit parameters.
- Each defined variable must have an assigned number. This number will substitute for the variable in the equation.
- Always start a formula with G as the dependent variable in the form of G(x)=, x being any arabic letter.
- The independent variable x is considered to have the SI unit [s] and the same holds true for any dependent parameter to it.
- If there are no syntax errors, parameters and operations will be indicated.

14.1.2.2.5.4 Model Equations

The acquired correlation functions must be fitted to models in order to retrieve meaningful results. It depends on the biochemical process, which model is the most appropriate. If the underlying process is known, the model can be selected prior to the start of the experiment. For example, if diffusion in a membrane is the subject, a 2-D diffusion model should be applied. In other cases, the process is not known, for example for free or anomalous diffusion. In this case, one can screen different potential models and look for the best fit taken into account the χ^2 value. Often two models work nearly equally well, for example, a two component free diffusion model can give you as satisfactorily a fit as a one component anomalous diffusion model, and without prior knowledge about the system it will be impossible to decide, which is the better one. In principle, models can be excluded if the fit does not work. However, a working model is only a potential candidate but does not signify it to be the correct one. Care should be taken to minimize the free parameters as much a possible to improve on the fit quality. It is not advisable to fit to three components without fixing the parameters of at least one of them. For example, if the diffusion time of a free ligand can be determined in a pre-experiment, that value should be fixed to reduce the number of floating parameters for the evaluation of the binding experiment to its receptor.

The FCS software is designed to be flexible. That means that the user can define or assemble equations which are useless. Care should be taken and formulas should be compared to the ones known from literature to obtain meaningful results. Also, the presence of a model does not necessarily mean, that the quality of the recorded data allows its usage. For example, anti-bunching requires a lot of care in data acquisition like long measurement times and cross-correlation to reduce dead times of the detectors and elimination of after-pulsing artefacts. It is in the responsibility of the user to set up his or her experiments accordingly.

The correlation function

Definition of the auto-correlation function

$$G^{\delta I}(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t+\tau) \rangle}{\langle \delta I(t) \rangle^2} = \frac{\frac{1}{T} \cdot \int_0^T (\delta I(t) \cdot \delta I(t+\tau))dt}{\frac{1}{T^2} \cdot \int_0^T (\delta I(t))^2 dt} = \frac{T \cdot \int_0^T (\delta I(t) \cdot \delta I(t+\tau))dt}{\int_0^T (\delta I(t))^2 dt}$$

or

$$G^{I}(\tau) = \frac{\langle I(t) \cdot I(t+\tau) \rangle}{\langle I(t) \rangle^2} = \frac{\frac{1}{T} \cdot \int_0^T (I(t) \cdot I(t+\tau))dt}{\frac{1}{T^2} \cdot \int_0^T (I(t))^2 dt} = \frac{T \cdot \int_0^T (I(t) \cdot I(t+\tau))dt}{\int_0^T (I(t))^2 dt}$$

where $\langle \, \rangle$ denotes the time average and $\delta I(t) = I(t) - \langle I(t) \rangle$ describes the fluctuations around the mean intensity.

For long time average of 1 (no bleaching) the following relation exists:

$$G^{I}(\tau) = 1 + G^{\delta I}(\tau)$$

Definition of the cross-correlation function

The formalism for the cross-correlation function is identical to the auto-correlation function, with the exception that the signal in one channel is not compared to itself, but to a signal in a second channel. Lets assign the indices "r" and "b" for the red and blue channel, respectively, than the cross-correlation function would read as follows:

$$G_X^{\delta I}(\tau)=\frac{\langle \delta I_b(t)\cdot \delta I_r(t+\tau)\rangle}{\langle I_b(t)\rangle\cdot\langle I_r(t)\rangle}=\frac{\langle \delta I_r(t)\cdot \delta I_b(t+\tau)\rangle}{\langle I_b(t)\rangle\cdot\langle I_r(t)\rangle}$$

$$G_X^{I}(\tau)=\frac{\langle I_b(t)\cdot I_r(t+\tau)\rangle}{\langle I_b(t)\rangle\cdot\langle I_r(t)\rangle}=\frac{\langle I_r(t)\cdot I_b(t+\tau)\rangle}{\langle I_b(t)\rangle\cdot\langle I_r(t)\rangle}$$

Note that the software for FCS calculated G^I functions, which do therefore converge to 1 and not 0. The acquired correlation functions are than compared to model equations.

Available model equations

In the following available equations used for the fits are given that define the accessible parameters. For some equations useful conversions to other parameters are listed as well. The total correlation is given by equation 2:

$$G_{tot}^{I}(\tau)=1+d+B\quad +A\cdot\prod_k\sum_l G_{k,l}(\tau)$$

where **d** is the offset, **B** the background correction, **A** the amplitude and $\mathbf{G_{k,l}(\tau)}$ the correlation for a single process. The suffixes k and l signify correlation terms for dependent and independent processes, respectively, that are multiplied with or added to each other.

The total correlation is therefore the amplitude multiplied to the product of the single correlation terms that are dependent and hence convolute each other. This amplitude has to be corrected for background and any offset. In cases, when the processes are independent from each other, the single correlations terms add up, for example in cases where there is more than one component all bearing the same label or of bunching terms that are independent from each other. If independent and dependent processes are present, all independent terms will add up and are multiplied with the dependent terms.

One can distinguish between different classes of fluctuation processes: anti-bunching, bunching and diffusion.

14.1.2.2.5.4.1 Amplitudes

The amplitude of the correlation function is influenced by the offset, background and the number of particles in dependence of the geometric factor. The amplitude is also influenced by the process of correlation.

The "1"

In a normal correlation, the curve converges to 1, in case intensities **I** are correlated as is the case with the FCS software. Note that in other cases, if fluctuations Iδ are correlated, the correlation function converges to 0, if no bleaching occurs.

$$G^{\delta I}(\tau)=1+G^{I}(\tau)$$

You can therefore easily convert $G^I(\tau)$ to $G^{\delta I}(t)$ values by adding a fixed offset of –1.

Offset d

$$d = c$$

c is any rational number that can be negative or positive.

In the FCS software the offset can be a fit parameter or a fixed value. In some cases, especially if very slow or immobile components are present, there can be a positive offset from 1. This offset can be taken into account by fitting to d. On the other hand, if the offset is known, it can be fixed. The offset will be added to each correlation value.

Background B

$$B = (1 - \frac{I_b}{I_t})^2$$

where I_b is the background intensity and I_t is the total intensity.

The FCS software treats the background always as a fixed value and never as a fit parameter. Thus the user has to define the background.

Note that the background in this case refers to a non-correlating background. If there is no background intensity, $B = 1$, otherwise $B < 1$. The background can be determined by measuring an unlabeled solution or cell at the same settings than the real experiment and recording the count rate Ib. The real experiment with the labeled species will give It. A non-correlating background will result in a lower amplitude and hence overestimation of molecule numbers, if not corrected for. Note the squared correction term.

Amplitude A

$$A = \frac{\gamma}{N} = G(0) - 1$$

where γ is the geometric factor accounting for the point spread function (PSF) and N the mean number of particles.

In the FCS software γ can be a fit or a predefined fixed value. In case γ is a fit value N must be fixed in the fit procedure. N is normally a fit parameter.

Please note that γ takes different values for different fitting models depending on the assumed intensity distribution of the point spread function (PSF):

γ_C=1.000 (cylindrical)
γ_{2DG}=0.500 (2-D Gaussian)
γ_{3DG}=0.350 (3-D Gaussian)
γ_{GL}=0.076 (Gaussian-Lorentzian).

γ can also be calibrated, if a known concentration c of a dye is measured. In this case N can be fixed and γ fitted. The obtained number can be entered as the calibrated fixed number. N can be calculated from equation

$$c = \frac{N}{V \cdot L_A}$$

with V being the confocal volume and $L_A = 6.023 \times 10^{23}$ mol^{-1} the Avogadro number.

The volume V is calculated from equation

$$V = \pi^{3/2} \cdot \omega_r^2 \cdot \omega_z$$

with ω_z axial focus radius and ω_r the lateral focus radius. The radii themselves have to be determined by a calibration measurement using a dye with a high quantum yield and a known diffusion coefficient D from the fitted diffusion time τ_d and the structural parameter S employing a free diffusion model with triplet state.

The following relations exist:

$\tau_d = \frac{\omega_r^2}{4 \cdot D}$ for 1 photon excitation

$\tau_d = \frac{\omega_r^2}{8 \cdot D}$ for 2 photon excitation

$S = \frac{\omega_z}{\omega_r}$

Equations 5e or 5f, dependent on the excitation source, can be used to retrieve ω_r; with its knowledge ω_z can be calculated from equation 5g.

"N" can have different meanings in different fit models. For biology, normally the number of diffusing particles is of interest. In this case, if photo-physical processes (triplet, blinking, stretched exponentials) are involved, it is recommended to use their normalized forms, since then the number of molecules correspond directly to the number of diffusing particles. If photo-physical terms are not normalized, the number measured is the total number of diffusing particles and those undergoing photo-physical processes.

14.1.2.2.5.4.2 Anti-bunching terms

Anti-bunching is the phenomenon that a molecule cannot produce emitted photons as long as it stays in the excited state. Hence during the transition time required to drop back to the ground state, which corresponds in most of the cases to the lifetime if no other photo-physical processes are involved, no photon can be expected, which results in anti-correlation and hence a drop of the correlation function below 1.

Independent to other terms

$$G_a(\tau) = (1 - C - C \cdot e^{-\tau/\tau_a})$$

$$G_a(\tau) = (1 - \frac{C \cdot e^{-\tau/\tau_a}}{1 - C})$$

where C is the amplitude and τ_a the transition time, also referred to as the lifetime.

Dependent in combination with other terms

$$G_a(\tau) = (-C \cdot e^{-\tau/\tau_a})$$

where C is either a fit parameter or a fixed value and often takes the value 9/5.

There are two cases to be distinguished: First, if the anti-bunching is independent with other processes, than equations 6a and 6b in the non-normalized or normalized form must be used and the terms are multiplied with other correlation terms. In case the anti-bunching is treated dependent to other processes, than equation 6c is the correct one to use and the term is added to other correlation terms.

Stretched exponential – anti-bunching

This is a more general term adding frequency and stretched factors to the exponent.

$$G_k(t) = 1 - K_1 - K_1 \cdot e^{(-k_1 \cdot \tau/\tau_{k1})^{\kappa 1}} \quad \text{not normalized}$$

$$G_k(t) = 1 - \frac{K_1 \cdot e^{(-k_1 \cdot \tau/\tau_{k1})^{\kappa 1}}}{1 - K_1} \quad \text{normalized}$$

where K_1 is the fraction of molecule, and τ_{k1} the exponential decay time, k_1 the frequency factor and κ_1 the stretch factor.

K_1 and τ_{k1} are fit parameters; k_1 is a fixed parameter and must be user defined; κ_1 is either a fit parameter or can be fixed.

Note, fixing k_1 and κ_1 to "1" results in a simple anti-bunching term.

Double stretched exponential – anti-bunching

This is a double exponential function, where the exponentials are subtracted.

$$G_k(t) = 1 - K_1 - K_1 \cdot e^{(-k_1 \cdot \tau/\tau_{k1})^{\kappa 1}} - K_2 - K_2 \cdot e^{(-k_2 \cdot \tau/\tau_{k2})^{\kappa 2}} \quad \text{not normalized}$$

$$G_k(t) = 1 - \frac{K_1 \cdot e^{(-k_1 \cdot \tau/\tau_{k1})^{\kappa 1}} + K_2 \cdot e^{(-k_2 \cdot \tau/\tau_{k2})^{\kappa 2}}}{1 - K_1 - K_2} \quad \text{normalized}$$

where K_1 and K_2 are the fractions of molecules, and τ_{k1} and τ_{k2} the exponential decay times, k_1 and k_2 the frequency factors and κ_1 and κ_2 the stretch factors.

K_1, K_2, τ_{k1} and $\tau_{k2}$are fit parameters, k_1 and $k_2$are fixed parameters and must be user defined, κ_1 and $\kappa_2$are fit parameters or can be fixed.

14.1.2.2.5.4.3 Bunching terms

Bunching is the phenomenon of a burst of photons during a certain time interval, the duration of which is determined by photo-physical processes including triplet, blinking, flickering and protonation. These terms are exponential decay functions. Formally, they look the same, only the exponential decay might be different.

Triplet

$$G_t(\tau) = (1 - T_t + T_t \cdot e^{-\tau/\tau_t}) \quad \text{not normalized}$$

$$G_t(\tau) = (1 + \frac{T_t \cdot e^{-\tau/\tau_t}}{1 - T_t}) \quad \text{normalized}$$

where T_t is the triplet fraction, that is the number of molecules undergoing triplet states and τ_t the triplet decay time.
T_t and τ_t are fitted parameters.

Triplet is based on an un-allowed intersystem crossing from the excited to the so-called triplet state. This state lasts for 1 – 5 µs. If the electron drops back to the ground state, no photon is emitted and hence during the triplet state the molecule is in a dark state. Triplet is indicated as a rise in the correlation amplitude, which is indicated as a deviation from the flattening curve at shorter correlation times. If not normalized, the triplet fraction contributes to the total number of molecules.

Blinking

$$G_b(\tau) = (1 - T_b + T_b e^{-\tau/\tau_b})$$ not normalized

$$G_b(\tau) = (1 + \frac{T_b \cdot e^{-\tau/\tau_b}}{1 - T_b})$$ normalized

where T_b is the blinking fraction, that is the number of molecules in the dimmer state and τ_b the blinking decay time of the dimmer state. Note, if the blinking term is not normalized, the number of blinking molecules will influence the total number of molecules.
T_b and τ_b are fitted parameters.

Blinking is based on the phenomenon that the electron distribution over conjugated systems can change in dependence on the local environment, for example changes in the pH, which will lead to molecules in a bright and dim or dark state. It is therefore a kinetic process that can be described in the following way with the following relations:

$$B \xleftrightarrow{k_F ; k_R} D$$

$$\tau_b = \frac{1}{k_F + k_R}$$

$$T_b = \frac{k_F \cdot k_R \cdot (\eta_B - \eta_D)^2}{(k_F + k_R) \cdot (k_F \cdot \eta_B^2 + k_R \cdot \eta_D^2)}$$ with the constraint $\eta_B > \eta_D$

with B and D representing the brighter and darker states, k_F and k_R the forward and backward reaction rates and η_B and η_D the emission yields or molecular brightness of molecule species (D or B) in Hz or the relative dimensionless brightness. In case, where darker state is completely dark (η_D=0), equation 7h simplifies to

$$T_b = \frac{k_R}{k_F + k_R}$$

Note that Blinking is referred to a process that does not lead to a covalent modification in the chemical bonds. If covalent changes occur the process is referred to as Flickering, which is formally treated in the same way.

Dependent triplet and blinking

In this case the terms are just representatives for two dependent bunching terms that are linked by addition (double exponential term). Note that the triplet fraction, if present, could be potentially fitted to either of the terms.

$$G_t(\tau) = (1 - T_1 + T_1 \cdot e^{-\tau/\tau_{t1}} - T_2 + T_2 \cdot e^{-\tau/\tau_{t2}})$$ not normalized

$$G_t(\tau) = (1 + \frac{T_1 \cdot e^{-\tau/\tau_{t1}} + T_2 \cdot e^{-\tau/\tau_{t2}}}{1 - T_1 - T_2})$$ normalized

where T_1 and T_2 are the fractions of molecules in the triplet state, and τ_{t1} and τ_{t2} the triplet exponential decay times.
T_1, T_2, τ_{t1} and $\tau_{t2}$are all fitted parameters.

Independent triplet and blinking

In this case the terms are just representatives for two dependent bunching terms that are linked by multiplication. Note that the triplet fraction, if present, could be fitted to either of the terms.

$$G_t(\tau) = (1 - T_1 + T_1 \cdot e^{-\tau/\tau_{t1}})(1 - T_2 + T_2 \cdot e^{-\tau/\tau_{t2}})$$ not normalized

$$G_t(\tau) = (1 + \frac{T_1 \cdot e^{-\tau/\tau_{t1}}}{1 - T_1})(\frac{1 + T_2 \cdot e^{-\tau/\tau_{t2}}}{1 - T_2})$$ normalized

where T_1 and T_2 are the fractions of molecules in the triplet state, and τ_{t1} and τ_{t2} the triplet exponential decay times.
T_1, T_2, τ_{t1} and $\tau_{t2}$are all fitted parameters.

Stretched exponential - bunching

In some reactions the kinetics cannot be fitted to simple exponential functions but require stretched exponentials.

$$G_k(t) = 1 - K_1 + K_1 \cdot e^{(-k_1 \cdot \tau/\tau_{k1})^{\kappa 1}}$$ not normalized

$$G_k(t) = 1 + \frac{K_1 \cdot e^{(-k_1 \cdot \tau/\tau_{k1})^{\kappa 1}}}{1 - K_1}$$ normalized

where K_1 is the fraction of molecule, and τ_{k1} the exponential decay time, k_1 the frequency factor and κ_1 the stretch factor.
K_1 and τ_{k1} are fit parameters, k_1 is a fixed parameter and must be user defined, κ_1 is either a fit parameter or can be fixed.

Note, fixing k_1 and κ_1 to "1" result s in a simple bunching term.

Double stretched exponential - bunching

This is a double exponential function, where the exponentials are added.

$$G_k(t) = 1 - K_1 + K_1 \cdot e^{(-k_1 \cdot \tau/\tau_{k1})^{\kappa 1}} - K_2 + K_2 \cdot e^{(-k_2 \cdot \tau/\tau_{k2})^{\kappa 2}}$$ not normalized

$$G_k(t) = 1 + \frac{K_1 \cdot e^{(-k_1 \cdot \tau/\tau_{k1})^{\kappa 1}} + K_2 \cdot e^{(-k_2 \cdot \tau/\tau_{k2})^{\kappa 2}}}{1 - K_1 - K_2}$$ normalized

where K_1 and K_2 are the fractions of molecules, and τ_{k1} and τ_{k2} the exponential decay times, k_1 and k_2 the frequency factors and κ_1 and κ_2 the stretch factors.
K_1, K_2, τ_{k1} and τ_{k2} are fit parameters, k_1 and k_2 are fixed parameters, κ_1 and κ_2 are fit parameters or can be fixed.

This term is often required to fit protonation, with the second stretch factor and the frequency factors are set to "1".

14.1.2.2.5.4.4 Diffusion terms

Diffusion is driven by Brownian motion. We can distinguish translational, rotational and flow diffusion.

Rotational diffusion

In the most general form, rotation can be described as the sum of 5 exponential terms

$$g_r(\tau) = 1 - R_a + R_a \cdot \sum_{m=1}^{5} c_m \cdot e^{-r_m \cdot \tau / \tau_{r,m}}$$

where R_a is the amplitude, c_m the relative amplitude, r_m the frequency factor and $\tau_{r,m}$ the rotational diffusion time.

However, there are special cases that are of more use. In symmetric rotation, the general formula reduces to:

$$G_r(\tau) = 1 - R_a + R_a \cdot e^{-\tau/\tau_r}$$ not normalized

$$G_r(\tau) = 1 + \frac{R_a \cdot e^{-\tau/\tau_r}}{1 - R_a}$$ normalized

where R_a is the rotational amplitude and τ_r the rotational diffusion time.
R_a and $\tau_r$are fit parameters.

If rotation occurs dependent from other processes, the formula used as an additive term is defined as:

$$G_r(\tau) = R_a \cdot e^{-\tau/\tau_r}$$

R_a is either a fit parameter or a fixed value and often takes the value 4/5.

In case of asymmetric rotation, the term is as follows:

$$G_r(\tau) = 1 - R_a + R_a \cdot (c_1 \cdot e^{-r_1 \cdot \tau / \tau_{r,1}} + c_2 \cdot e^{-r_2 \cdot \tau / \tau_{r,2}})$$ not normalized

$$G_r(\tau) = 1 + \frac{R_a \cdot (c_1 \cdot e^{-r_1 \tau / \tau_{r,1}} + c_2 \cdot e^{-r_2 \tau / \tau_{r,2}})}{1 - R_a}$$ normalized

where R_a is the amplitude, c_1 and c_2 are relative amplitudes, r_1 and r_2 frequency factors, $\tau_{r,1}$ and $\tau_{r,1}$ rotational diffusion times.
R_a, $\tau_{r,1}$ and $\tau_{r,1}$ are fitted parameters, c_1, c_2, r_1 and r_2 are fixed values and must be user defined.

Rotational frequencies often take the following values:

r_1	1
r_2	10/3

The relative amplitudes dependent on the polarization of the excitation light and the analyzer in the emission beam path and are as follows:

Ex	lin. pol.	lin. pol.	lin. pol.	unpol.	unpol.	unpol.
Em	parallel	perpen-dicular	all	parallel	perpen-dicular	all
c_1	80	20/9	860/9	5/9	215/9	20
c_2	64/9	4	4	16/9	1	1

Translational diffusion

In its general form, translational diffusion is defined as:

$$G_d(\tau) = \left(\sum_{i=1}^{3} \frac{\Phi_i}{\left(1+\left(\tau/\tau_{d,i}\right)^{\alpha_i}\right)^{e_{d1}} \cdot \left(\left(1+\left(\tau/\tau_{d,i}\right)^{\alpha_i} \cdot 1/S^2\right)^{1/2}\right)^{e_{d2}}} \right)$$

with the constraint $\sum_i \Phi_i = 1$

where $\tau_{d,i}$ is the diffusional correlation time of molecule species i, S the structural parameter that is the ratio of axial to lateral focus radii, α_i the anomaly parameter or temporal component of molecule species I, $e_{d1}$and e_{d2} are fixed values and have to be user defined. The following values define 1-, 2-and 3-D diffusion:

e_{d1}	e_{d2}	dimensionality
1/2	0	1-D
1	0	2-D
1	1	3-D

Note that in the FCS software these values are automatically selected with the choice of dimensionality.

S is either a fit parameter or a fixed value. It is an instrumental parameter and can be determined by a calibration experiment using a dye solution with a known diffusion as a fit result.

α_i is either a fitted value for anomalous diffusion or a fixed value (set to "1") for free diffusion. The following relation exists:

α	Diffusion process
= 1	Free diffusion
< 1	Anomalous sub-diffusion
> 1	Anomalous super-diffusion

Note that α_i is set automatically to "1", if free diffusion is selected. If anomalous diffusion is selected, the parameter will float.

$\tau_{d,i}$ are fitted parameters. They can be converted to diffusion coefficients D_i using formulas 5e or 5f. The FCS software allows you to directly fit to D_i values, but in this case the lateral radius ω_r has to be specified as a fixed value.

Please note that in the case of anomalous diffusion the following relations exist:

$$\tau^{\alpha}{}_{d,i} = \frac{\omega_r^2}{\Gamma_{2,i}}$$

$$\tau^{\alpha}{}_{d,i} = \frac{\omega_r^2}{2 \cdot \Gamma_{2,i}}$$

where Γ is the transport coefficient of the fractional time dimension.

Please note the following relation between D and G:

$$D(t) = \Gamma \cdot t^{\alpha - 1}$$

If activating the fitting to the diffusion coefficient the Γ values have to be calculated from the D values by the following conversion:

$$\Gamma = 4 \cdot D$$

The Φ_i values are fit parameters. They account for different brightness of different components. In principle, if molecules of different brightness are present, the apparent molecular brightness is defined as

$$\eta = \frac{\sum_{i=1}^{3} f_i \cdot \eta_i^2}{\sum_{i=1}^{3} f_i \cdot \eta_i}$$

where η_i is the brightness of the molecules in kHz or the dimensionless relative brightness values. The brightness of the species has to be determined beforehand in control experiments.

Note that the brightness contributes as the square to the correlation function, in other words a double as bright molecule will contribute 4 fold more. Therefore, the fitted number of molecules must be corrected to obtain the real number N_{diff} of diffusing particles; please note that to obtain the diffusing particle number directly, other terms should be used in their normalized form:

$$N_{diff} = N \cdot \frac{(\sum_{i=1}^{3} f_i \cdot \eta_i)^2}{\sum_{i=1}^{3} f_i \cdot \eta_i^2}$$

If one wants to know the true fraction f_i of each species, values those can be retrieved with the known brightness from the relation

$$\Phi_i = \frac{f_i \cdot \eta_i^2}{\sum_{i=1}^{3} f_i \cdot \eta_i^2}$$ with the constraints $\sum_i \Phi_i = 1$ and $\sum_i f_i = 1$

If there is no brightness difference between the components, Φ_i will become f_i.

You can fit directly to the fractions even in case of different brightness values. In this case, the brightness values have to be fixed parameters and defined by the user. The fit formula converts from equation 5a in combination with 8g taking into account the corrected amplitude to equation 8o:

$$G_{tot}(\tau)=\frac{\gamma}{N}\cdot\frac{\sum_{i=1}^{3} f_i\cdot\eta_i^2}{(\sum_{i=1}^{3} f_i\cdot\eta_i)^2}\cdot\left(\sum_{i=1}^{3}\frac{\frac{f_1\cdot\eta_1^2}{\sum_{i=1}^{3} f_i\cdot\eta_i^2}}{\left(1+\left(\tau/\tau_{d,i}\right)^{\alpha_i}\right)^{e_{d1}}\cdot\left(\left(1+\left(\tau/\tau_{d,i}\right)^{\alpha_i}\cdot 1/S^2\right)^{1/2}\right)^{e_{d2}}}\right)=\frac{\gamma}{N}\cdot\left(\sum_{i=1}^{3}\frac{\frac{f_1\cdot\eta_1^2}{(\sum_{i=1}^{3} f_i\cdot\eta_i)^2}}{\left(1+\left(\tau/\tau_{d,i}\right)^{\alpha_i}\right)^{e_{d1}}\cdot\left(\left(1+\left(\tau/\tau_{d,i}\right)^{\alpha_i}\cdot 1/S^2\right)^{1/2}\right)^{e_{d2}}}\right)$$

with fixed brightness values η_i.

Flow

Flow signifies active transport either via cytoplasmic movement or directed transport.

If flow occurs in the absence of translational diffusion, the term is defined as follows:

$$G_f(\tau)=e^{-\left(\tau/\tau_f\right)^2}$$

In the presence of translational diffusion, the term alters to:

$$G_f(\tau)=e^{-\frac{(\tau/\tau_f)^2}{1+(\tau/\tau_d)}}$$

with τ_f representing the average residence time for flow and τ_d the diffusion correlation time.

Note, in the FCS software, the correct term is automatically loaded in dependence on the absence or presence of a translational term.

With the knowledge of the lateral radius ω_r given as a fixed value, the software allows to fit directly to the velocity v instead of the average residence time. The following relation exists:

$$v=\frac{\omega_r}{\tau_f}$$

14.1.2.2.5.5 Terms and Term Settings

Terms

Start the definition of a model by activating the checkbox of the term you want to include into the final correlation equation. Deactivate the checkbox to remove the term from the final equation. All activated terms are assembled into the final equation that is displayed in **G(τ)=** display area.

Choose from:

Term	Description
Offset d	Deviation from 1
Background B	Unspecific non-correlating background
Amplitude A	Number of molecules and geometric factor
Antibunching $G_a(\tau)$	Antibunching correlation term

Term	Description
Triplet $G_t(\tau)$	Exponential correlation terms for triplet state, blinking, flickering or other bunching correlation terms
Rotation $G_r(\tau)$	Rotational diffusion correlation term
Translation $G_d(\tau)$	Translational diffusion correlation term
Flow $G_f(\tau)$	Flow correlation term
Stretched exponential $G_e(\tau)$	Stretched exponential correlation term for kinetics

Settings

For a selected term the **Settings** button becomes available. The **Settings** panel allows you to choose specific equations and assign values to parameters, if applicable. The **Settings Description** box provides information on the parameter or displays the formula of the equation. In addition it provides useful conversions of the fitted parameter to other interesting parameters. For more information the used formula see *Model Equations [▸ 942]*.

The following settings are available:

Term	Settings
Offset d	▪ Set the offset to 0 by clicking the **Normalized** button. ▪ Define the offset by clicking the **Calibrated** button Type in a value into the edit box or set a value using the scroll arrows. ▪ The offset can have positive or negative numbers and the value specified will be added to all correlation values. ▪ The description panel provides information on the offset.
Background B	▪ Set an offset by typing in value in the **Background** selection box. or use the scroll arrows. ▪ If there is no background selected, the background correction factor is set to 1.
Amplitude A	▪ Set a value for the geometric factor γ, which describes the point-spread function. ▪ By clicking the **cylindrical**, **2DG**, **3DG** and **GL** buttons a cylindrical, 2 dimensional Gaussian, 3 dimensional Gaussian (3DG) and Gaussian-Lorentzian (GL) PSF can be set, respectively. ▪ click the **Calibrated** button to type in a user defined number into the edit box or use the scroll arrows to set a value. ▪ The description panel provides information on the amplitude. ▪ At least one the γ factor or the number of molecules N have to be fixed in the fit procedure.
Antibunching $G_a(\tau)$	▪ Choose between normalized and non-normalized anti-bunching term by activating / deactivating **Normalized**. ▪ Select the **Dependent antibunching, rotation and translation** form by checking the respective box. This equation is used as an additive term to rotational and translational diffusion terms.

Term	Settings
	- Leave the amplitude value free to float or to set the value to 9/5 by clicking the **Free** or **9/5** buttons, respectively. The active selection will be highlighted in blue. - The description panel provides information on the anti-bunching.
Triplet $G_t(\tau)$	The triplet represents bunching terms that are exponential decay functions. - Choose between normalized and non-normalized triplet term by activating / deactivating **Normalized**. - Choose, whether the bunching terms should be weighted or not by activating or deactivating **Weighted**. If no weights are applied, the fit follows the measured curve to minimize χ^2. If no boundary values are set for the relaxation times, noise might also be followed and triplet fractions might show up to be too high. If weights are applied, noise is followed less. The following weight equation is used: $Weight(t) = 1 + 10^{-t(t)/3.2\times10^{-6}}$ Several options are available for the bunching terms. Select the relevant term from the **Components** drop down menu: - Triplet: 1 exponential function - Blinking: 1 exponential function - Independent Triplet and Blinking: Sum of 2 exponential functions - Dependent Triplet and Blinking: Product of 2 exponential functions - The name of the selected term is shown in the **Components** selection box. - The description panel provides information on the bunching terms.
Rotation $G_r(\tau)$	- Choose between normalized and non-normalized rotation functions by activating / deactivating **Normalized**. - Select the **Dependent antibunching, rotation and translation** by activating the respective checkbox. This equation will be used as an additive term to anti-bunching and translational diffusion terms. Optionally leave the amplitude value free floating or setting it to 4/5 by clicking the **Free** or to **4/5** buttons, respectively. The active option is highlighted in blue. - Choose between symmetric rotation (1 exponential term) and asymmetric rotation (double exponential term) by activating / deactivating **Asymmetric**. When **Asymmetric** is active, it is possible to define relative amplitudes and rotational frequencies (note, these parameters are no fit values and have to be defined by the user). Relative amplitudes are defined by selecting the excitation polarization (**linear polarized** or **unpolarized**) and the emission detection (**parallel**, **perpendicular** or **all**), by clicking the corresponding tabs, which will result in the corresponding values displayed in the **c1** and **c2** display boxes. Alternatively, if **others** is activated, you can type in user defined values. Rotational frequencies can be selected by clicking the **Default** tab to load the default values. The corresponding values are shown in the **r1** and **r2** display boxes. By clicking the **other** tab, user defined values can be entered.

Term	Settings
	▪ The description panel provides information on the rotational diffusion terms.
Translation $G_d(\tau)$	The following possibilities for parameter settings are available: ▪ Fit to fractions (normally used when no brightness differences are observed between different components) or to fractional intensities, by activating **Fractional Intensities**. Type in the absolute or relative brightness values of the components into the **Molecular brightness** edit box. ▪ Fit to the diffusion time, or directly fit to the diffusion coefficient by activating**Diffusion coefficients**. Type in the radial dimension of the confocal volume into the ω_r edit box or use the scroll arrows. ▪ In case two photon excitation is used activate **2 Photon** since this will influence the fit formula in the case the **Diffusion coefficients** option was chosen. ▪ Select the number of components (1, 2 and 3) by clicking the Components **1**, **2** or **3** buttons. The number of active components will be highlighted in blue. ▪ Select **free** / **anomalous** diffusion in the dropdown down menus beneath each component button. ▪ Set the diffusion **Dimension** in the dropdown menus beneath each component button. Select between 1-D, 2-D and 3-D. ▪ Enter brightness values in the **Brightness** edit boxes for each component. These values are only displayed, if the **Fractional Intensities** option is selected. Type in absolute values (that must have the same units for all components) or relative values. ▪ The description panel provides information on the translational diffusion terms.
Flow $G_f(\tau)$	▪ Determine, whether you want to fit to the diffusion time or directly to the velocity by activating **Velocity**. Type in the radial dimension of the confocal volume into the ω_r edit box or use the scroll arrows. ▪ The description panel provides information on the flow terms. ▪ Note, that the system automatically toggles between the pure flow and flow in combination with translational diffusion in cases where the **Translation** term is deactivated or activated, respectively.
Stretched exponential $G_e(\tau)$	▪ Choose between bunching and anti-bunching terms by clicking the **Bunching** or **Antibunching** buttons, respectively. ▪ Choose between 1 (mono exponential) or 2 (double exponential) stretched exponential terms by clicking the **Components 1** or **2** buttons. The active option will be displayed in blue. ▪ Choose between normalized and non-normalized stretched exponential term by activating / deactivating **Normalized**. ▪ Choose between dependent and independent stretched exponentials by choosing from the **Components** dropdown menu. This becomes only available, if two components are active. ▪ The description panel provides information on the strethed exponential terms.

Term	Settings
	▪ Note, the frequencies and stretch factors are no fit parameters and must be defined in the **Frequencies k1** and **k2** as well as the **Stretch factors κ1** and **κ2** display boxes. Either enter a value or click the **1** button for the default setting. ▪ For two components, only the formula for dependent processes is presently available.

14.1.2.3 Setting up FCS/FCCS Measurement

14.1.2.3.1 FCS Measurement Setup for Labeled Molecules in Solution

Prerequisite

- ✓ Dye/Molecule dissolved in suitable solvent and at suitable concentration.
- ✓ High NA Objective selected for imaging.
- ✓ Focus point set into the solvent maybe using imaging techniques to check on emission signal.

1. Chose **FCS track** in **Imaging Setup** tool.
2. Select detection channel for FCS in **Imaging Setup** tool.
3. Select laser and laser power for dye excitation in **Channels** tool (see *Choosing the Optimal Laser Power [▸ 958]*).
4. Select 1 Airy Unit for the pinhole.
5. Set up measurement parameters in **Channels** tool (see *Setting the Parameters for the FCS/ FCCS Measurement [▸ 958]*).
6. Click **Continuous** for continuous measurement to see count rates and correlation data and to optimize laser power.
7. Adjust Pinhole if necessary (i.e. if low count rate is registered during continuous data acquisition with otherwise sufficient laser power) (see *Adjusting the Pinhole [▸ 959]*).
8. Click **Snap** for a complete single dimensional measurement.

14.1.2.3.2 FCS Measurement Setup for Labeled Molecules within Cultured Cells

Prerequisite

- ✓ Dye/Molecule available/expressed in suitable concentration within cultured cell
- ✓ High NA Objective selected for imaging
- ✓ Pinhole adjusted with test sample previous to the measurement (if needed) (see *Adjusting the Pinhole [▸ 959]*

1. Focus onto the selected structure.
2. Acquire a confocal image using suitable imaging parameters (laser, laser power, detector gain, detection range) to identify the region/point of interest for FCS measurement.
3. Open **Experiment Regions** tool.
4. Select **FCS marker** tool and position it within the acquired image onto the point of interest.
5. Chose **FCS track** in **Imaging Setup** tool.
6. Select the required detection channel/s in **Imaging Setup** tool.
7. Select laser and laser power for dye excitation (see *Choosing the Optimal Laser Power [▸ 958]*).
8. Set up measurement parameters in **Channels** tool (see *Setting the Parameters for the FCS/ FCCS Measurement [▸ 958]*).

9. Click **Continuous** for continuous measurement to see count rates and correlation data and to optimize laser power and focus position.
10. If necessary run Z-Scan (count rate over Z stack measurement) to find optimal Z position or correct Z position using the Z Focus of the stand (see *Performing a Z-Scan [▸ 960]*).
11. Confirm the selected position within the **Experiment Regions** tool for the corresponding measurement spot.
12. Click **Snap** for a single complete measurement including all selected measurement spots.

14.1.2.3.3 FCS Measurement Setup for Labeled Molecules in Solution Using a Sample Carrier

Prerequisite

- ✓ Dye/Molecule dissolved in suitable solvent and at suitable concentration distributed into a sample carriers wells.
- ✓ High NA Objective selected for imaging.
- ✓ ZEN Module **Tiles & Positions** is available on the system.
- ✓ Focus point set into the solvent (about 100 to 200 microns above the coverslip into the solvent) maybe using imaging techniques to check on emission signal.
- ✓ FCS track is defined with suitable channel selection, laser line and power, and measurement parameters for the actual FCS measurement.
- ✓ Pinhole is adjusted for optimal FCS results.

1. Activate an LSM confocal track (or other imaging track).
2. Open **Tiles** tool.
3. Select the sample carrier matching the one used for the experiment.
4. Move the actual sample carrier to the reference point marked as yellow cross in the sample carrier graphic and confirm with ok to close the panel.
5. Calibrate the sample carrier with the following steps:
   - 1/4 -> no changes, click **Next**.
   - 2/4 -> Click **Set zero**, click **Next**.
   - 3/4 -> Chose **Search Reference Point** (1 Point) from the drop down list, click **Next**.
   - 4/4 -> Set **Current XY**, click **Finish**.
6. Activate FCS track .
7. Open **Tiles Viewer** and zoom out to see the carrier in total.
8. Select the following tool from the **Positions** icons on the left: (Setup new positions from an underlying sample carrier).
9. Mark the wells by clicking them indivually (*Click-Strg*) or select by drawing a contour around the relevant ones holding the left mouse button while moving the curser.
10. Keep the default settings: **Distribute Positions by Number**, **Number = 1**, and **Bias = None**.
11. Click **Plus** to add one position per marked well.
12. Click **Start Experiment** to acquire an FCS measurement per selected well.

Info

Only one position per carrier well is supported.

Repeated measurements per well can be achieved combining this type of acquisition with a Time series multidimensional acquisition.

Multiple Positions per well or tiles or combinations of tiles and positions are not supported and will be deleted when the FCS track is activated

If the sequence of the positions is changed after their initial definition the timely sequence of the position measurement data can only be deduced from the time stamp in the result table within the FCS document. IDs of positions and measurement data match.

14.1.2.3.4 Setting the Parameters for the FCS/FCCS Measurement

1. Set the **Measurement Time**.
   If you do not have any idea about the behavior of your sample, start with a measurement time of about 10 seconds. Depending on the signal-to-noise ratio of your correlation curve, decrease or increase the measurement time. As a rule of thumb the measurement time should be about 1000 x the diffusion time of a molecule under study. However, due to "photon noise" (photon shot noise) a 10 s measurement time is a good start value to obtain good statistical results.
2. Set the **Repetitions**.
   Start with **Repetitions** set to 1. If you want to obtain information about the variation of your fitted values later on, increase the Repetitions to produce a measurement series. 10 times is a good value to start with. Repetitions can be averaged into an average correlation function if required. This will improve statistics. An average of 10 x 10 seconds Measurement Time is not as good as one 100 sec measurement. If bleaching becomes a problem, it might help to reduce Measurement Time and increase Repetitions to obtain fairly good statistical results while reducing bleaching.

14.1.2.3.5 Choosing the Optimal Laser Power

1. Use the initially set laser power indicated in the **Laser control** panel in **Imaging Setup** or **Channels** Tool.
2. Start a **Continuos** scan with an active FCS track and bring the **Detector Counting** tool from the right tool area into view. A good starting point is a laser power which leads to a count rate between 50 kHz and 200 kHz.
3. Change the laser power using the slider next to the active laser used for acquisition to increase or decrease the values to achieve such a count rate.
4. When finished, stop **Continuous** scan.

Info

For most dyes, the **Counts / Molecule** setting should be optimized in a second step to a value just under its maximum by adapting the laser power. If carriers of different slide thickness are employed, the **Counts / Molecule** setting should be optimized by using the correction ring of the objective. The correction ring is turned counterclockwise or clockwise until a maximum value is obtained. The correction ring should also be used for adjusting the **Counts / Molecule** setting whenever the immersion media is changed. This is especially important in cases where the refractive index of the immersion media is different from that of the sample.

14.1.2.3.6 Adjusting the Pinhole

The pinhole is adjusted using a dye solution. For each excitation wavelength and MBS combination a suitable dye must be used.

We recommend:

- Rhodamine 6 Green (Rh6G) or Alexa 488 for excitation lines 445, 488 and 514 nm
- Tetra-Methyl-Rhodamine (TMR) or Alexa 546 for excitation line 543 nm or Alexa 568 for excitation line 561 nm
- Cy 5 or Alexa 633 for excitation line 639 nm

We recommend to work with a relatively concentrated solution (10^{-6} mol/l) and low laser power to achieve intensity curves with low noise.

Prerequisite ✓ All steps from *FCS Measurement Setup for Labeled Molecules in Solution [▸ 956]* up to the step Pinhole Adjust have been performed.

1. Set the laser power to achieve above 100 kHz count rate, if possible.
2. Click **Adjust** in the pinhole panel of the Channels tool.
3. Click the **Coarse** button of X first and then Y to perform a coarse adjustment scan in x and then in y.
   → The pinhole will travel over the maximum range for each axis.
   → The adjustment scan in y will use the optimum setting found for the x axis.
4. Perform a fine adjustment in x and y by subsequently clicking the **Fine** buttons.
   → For the fine adjustment the pinhole will travel only a limited distance (about 10 microns) around the peak determined in the coarse adjustment or around the current stored position of the pinhole if the coarse adjustment was skipped.
5. If the pinholes are adjusted for the first time, the coarse adjustment must be performed first followed by the fine adjustment, each time for x and y. For subsequent readjustments, the fine adjustment is typically sufficient.
6. In case more lasers and detectors are selected, the adjustment scan will be done for all lasers / detectors simultaneously.
7. When using the 405 nm laser it might be necessary to adjust the collimator for optimal overlay in Z. This should be done in advance to the pinhole adjust procedure if necessary (see *Collimators Adjustment [▸ 976]*). The collimating lens is pre-set at delivery and is a function of the wavelength and objective used. In case a new objective is used with the system this value needs to be determined anew.
8. The values determined by the adjustment scan for the X and Y position of the pinhole(s) are indicated by the position of the line in the graph as well as the slider and the number in the edit box. In case this value is not accepted it can be changed by moving the slider or typing in a different value.
9. Click finish to store the indicated X and Y values as general pinhole settings for the main dichroic used. This value will now be used for the current and future FCS / FCCS measurements.
   It is recommended to adjust the pinhole for FCS measurements on a regular basis.
10. The adjustment scan can be performed separately for X and Y. The values can be stored independently as well. However it is recommended to adjust both values and store them both.

14.1.2.3.7 Performing a Z-Scan

The Z-Scan is especially suitable to position the confocal volume on a cell membrane. Due to the shape of the confocal volume, the membrane should not be approached from the side but instead rather from the top of the cell. Please note that it is better to use the upper membrane for measurement, since the lower membrane might be too close to the glass bottom surface resulting in disturbing reflections. Besides using the **Z-Scan** you can also position the membrane manually, albeit with less precision.

Prerequisite

- ✓ Dye/Molecule available/expressed in suitable concentration within cultured cell.
- ✓ High NA objective selected for imaging.
- ✓ Pinhole adjusted with test sample previous to the measurement (if needed) (see *Adjusting the Pinhole [▶ 959]*).
- ✓ FCS track defined and active.
- ✓ Cell of interest positioned in the center of the scan field and manually focused as best as possible acquiring a confocal image.

1. In **Channels** tool, click **Z-Scan** button.
2. Set the range and step width for the scan suitable for the sample.
3. Start the Z-Scan.
   → The resulting graph should typically show two peaks (from the lower and upper membranes). High signal intensity may correlate to the labeled membranes.
   → If the glass surface happens to be in the Z-Scan range it will also give a peak. To determine its position, perform a Z-Scan at a position where there is no cell. If the glass reflection contributes so much that the peaks of the membranes are hidden due to scaling, shorten the scan range to avoid picking up the signal.
4. Place the red line at the peak that corresponds to the membrane and select this Z-position.
5. If no clear signal can be detected, or the peak of interest lies too close to the range extremes, take over the Z position most likely to represent the wanted one by moving the bar to this position and clicking **Finish**.
6. Restart the Z-Scan at this Z position with an adjusted range and step width and take over the then defined Z position for the FCS measurement.

14.1.2.3.8 Saving and Loading FCS Data

Whenever **Save FCS raw data file** is activated in the Acquisition Selection/LSM of the **Options** tool, the raw data file is saved along with the *.FCS file in the folder selected for saving the *.FCS file.

*.RAW files contain the same name than the *FCS.fcs file with name extensions, which identify the repetition and the channels.

The measurements can be saved individually using the standard save tools or by activating **Auto Save** in the left tool area. The *.FCS file includes the whole data set (curves, fitting results, fit parameters).

Whenever a *.FCS file is opened the data show up in the **Correlation view** tab of the FCS document (see *Correlation Tab [▶ 922]*).

The **Reload** function (see *Reload Tool [▶ 935]*) is only available if raw data have been saved along with the *.FCS file.

14.1.2.3.8.1 Format of *.RAW Files

*.RAW files are saved optionally when activating the storage of raw data in the options menu (see *Saving and Loading FCS Data [▸ 960]*). The *.RAW data files can be opened directly in ZEN. They are also opened automatically if linked to a *.FCS file. In this case all raw data files associated with the *.FCS file will be opened in the same FCS document.

Info

Raw data formats can also be opened in ZEN Black (all versions).

Info

The sampling rate is set to 15 MHz. Depending on the detector used for FCS measurements, a maximum data rate of up to 16 Mbyte/sec is achieved.

The recorded test has the following structure:

Bit	Meaning
0 (LSB) ... 7	▪ clock countervalue (starting at 1) during the triggering event ▪ trigger events are pulse recordings or counter overruns ▪ zero is reserved and only transmitted at the end of the measure-ment
8	1, if pulse recorded in channel 1 during cycle bt1; else 0
9	1, if pulse recorded in channel 2 during cycle bt1; else 0
10	1, if pulse recorded in channel 1 during cycle bt2; else 0
11	1, if pulse recorded in channel 2 during cycle bt2; else 0
12	1, if pulse recorded in channel 1 during cycle bt3; else 0
13	1, if pulse recorded in channel 2 during cycle bt3; else 0
14	1, if pulse recorded in channel 1 during cycle bt4; else 0
15 (MSB)	1, if pulse recorded in channel 2 during cycle bt4; else 0

Tab. 7: Structure of the recorded text

The following examples illustrate the format. The tables show part of the running pulse train (from left to right) divided into clock cycles. "1" in the corresponding box indicates that a pulse ar-rived in this cycle. The "counter" row shows the counter readings. The lowest row indicates when the text has been recorded.

				bt1	bt2	bt3	bt4							bt1	bt2	bt3	bt4		
CH 1				1		1						...							
CH 2					1							...							
counter	120	121	122	123	0	0	0	1	2	3	4	...	254	255	0	0	0	1	2
							⇑ W1										⇑ W2		

Example 1

				bt1	bt2	bt3	bt4							bt1	bt2	bt3	bt4		
CH 1				1		1						...			1				
CH 2												...				1			
counter	120	121	122	123	0	0	0	1	2	3	4	...	254	255	0	0	0	1	2
							⇑ W3										⇑ W4		

Example 2

Fig. 101: Examples of the recorded text

The following text is recorded:

Example 1:

- at W1: high byte: 00011001(bin) = 19 (hex); low byte: 123 (dec) = 7B (hex); resulting word = 7B19 (hex)
- at W2: high byte: 00000000(bin) = 00 (hex); low byte: 255 (dec) = FF (hex); resulting word = FF00 (hex)

Example 2:

- at W1: high byte: 00010001(bin) = 11 (hex); low byte: 123 (dec) = 7B (hex); resulting word = 7B11 (hex)
- at W2: high byte: 00100100(bin) = 24 (hex); low byte: 255 (dec) = FF (hex); resulting word = FF24 (hex)

Info

The first 30 bytes of the raw data file contain the comment "ConfoCor_x_-_Raw_data_file_1.0" and must be ignored.

The format is processed into one that describes the time distance between photons.

The format is explained in the following figure. The figure displays the raw data with an editor program that shows the raw data in file offset, hexadecimal, and as ASCII.

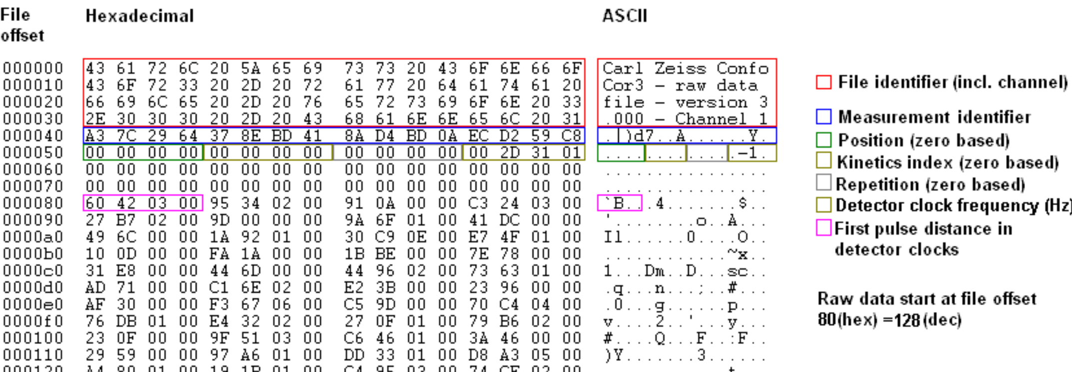


Fig. 102: ZEN raw data format

Bytes	Explanation
0-63	▪ represent the file identifier with the channel number
64-79	▪ measurement identifier The identifier is a randomly created number that will be assigned to all channels, repetitions, positions and kinetic indices of the same measurement. Hence, in a cross-correlation experiment, the two auto-correlation pairs that belong together can be identified.
80-83	▪ encode the position of the measurement ▪ zero based
84-87	▪ encode the kinetic index ▪ zero based
88-91	▪ encode the repetition number ▪ zero based
92-95	▪ encode the detector frequency in Hz
96-127	▪ reserved for comments ▪ set to 0
128-corresponding to a file offset of 7F (hex)=127 (dec)-to 131	▪ code for the first pulse distance in detector clocks
starting from 132	▪ code for follow up pulse distances

Tab. 8: Explanation of raw data code

14.1.3 Imaging Setup

14.1.3.1 LSM Confocal

Chose LSM confocal as acquisition track for standard confocal acquisition. The following channels are available for confocal acquisition:

- Ch1 = Multialkaline PMT (available for all configurations)
- ChS1 to ChS4 = GaAsP PMT (part of a 6 or 32 channel configuration)
- ChS5 to ChS8 = GaAsP PMT (part of a 32 channel configuration)
- Ch2 = Multialkaline PMT (part of a 6 or 32 channel configuration)
- Ch2 = GaAsP PMT (part of a 3 channel configuration)
- Ch3 = Multialkaline PMT (part of a 3 channel configuration)
- ChA = GaAsP PMT (optional, part of a configuration with)
- T-PMT = Mulitalkaline PMT (optional multialkaline PMT in transmission)
- GaAsP 1 and GaAsP 2 = GaAsP PMT (optional, detection module attached to DCout port)

Info

LSM confocal tracks can be used in a multitrack acquisition with either NDD (Non Descanned Detector) tracks or Airyscan SR tracks.

Fig. 103: Imaging Setup, LSM Confocal

1 **Switch track every**

Select the switch mechanism by which the hardware is changed between the tracks in case a multitrack acquisition is set up.

- **Line**

  Switches tracks in between every line of an image.

  This mode switches between the used laser line for each track and the detection channel(s) used for acquisition with their predefined gain and offset. Other hardware settings like filters, pinhole or the spectral width of a channel cannot be changed in between tracks.

  This mode is especially useful for applications with high temporal correlation between the different emission signals.

- Frame

  Switches tracks in between every full image frame.

  This mode allows to change all kinds of hardware in between tracks including main dichroic beam splitters, secondary beam splitters, emission filters for , filters in the reflector turret, the pinhole and the spectral width of the channel. This provides a highly specialized acquisition mode but with a slower overall acquisition.

- Frame Fast

  Switches tracks in between every full image frame (fast).

  This mode works similar to line wise switching with the difference that tracks are switched after each full image frame. The same restrictions as with line wise switching apply to parameter changes in between tracks. This mode reduces the frequency of sample illumination for each dye

- Z-Stack

  Switches tracks in between every full image stack.

  This mode is only available for image acquisition using Z stacks. The mode switches tracks in between every full Z stack acquisition. This reduces imaging time as switching between tracks is performed less often when for each image a frame wise multitrack is the best choice.

2 **Spectral display**

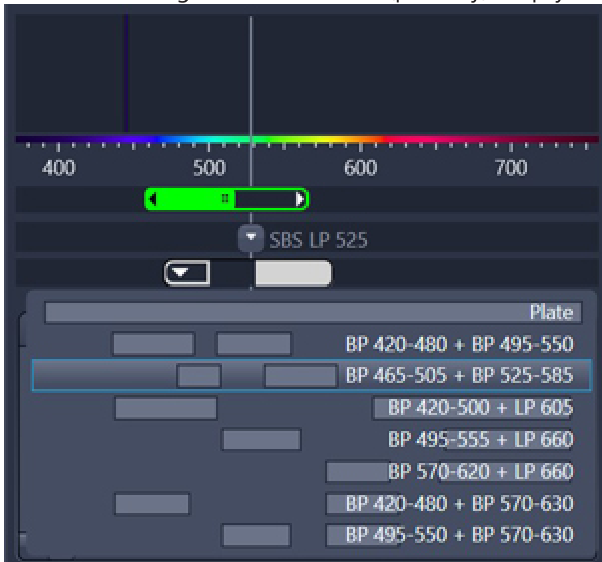
Shows the emission spectrum of the selected dye(s) assigned to active or non active channels of the displayed track and the laser lines used for excitation.

3 **Detection range**

A bar slider shows up for each activated channel. The slider color matches the color chosen for the corresponding channel. Channel 1 to Channel 3 are aligned from left to right. The width of the sliders represent the detection range covered by the respective channel. Change position of a slider with the left mouse button using the cross which appears when the cursor is moved over the slider. Accordingly, use the arrows at the edges of a slider to expand or shrink the width of the slider. Moving and adaption is only possible for internal channels. Channels with emission filters like or cannot be adapted in their spectral range unless a different filter is selected.

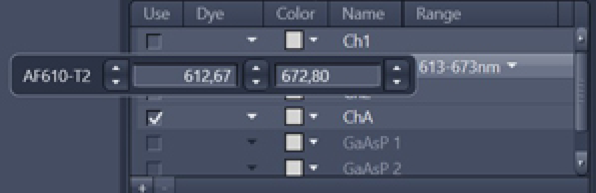
4 **Emission filter for Airyscan**

When Airyscan (ChA) is selected as detection channel the selection for Airyscan emission filters shows up in this line area. Chose a matching emission filter from the drop down list which opens up when clicking the arrow on the left hand side of the bar. As with the internal channels, the setting of the SBS filter affects the detection range of the Airyscan detector. This again is shown as a (partially) empty bar.



5 **Channel selection area**

This panel lists all available detection channels for **LSM Confocal** mode. Select / unselect checkbox Use for activation / deactivation of individual channels. Assign a dye to a channel by clicking the Dye drop down arrow and selecting a dye from the Add Dye or Contrasting Method Tool Window. Assig a color to the channel by clicking the arrow in the Color column and selecting a color from the LUT. The color can also be changed in the Channels tool. The name changes to the name of the dye if a dye is selected for this channel. The name can be changed in the Channels tool and then shows up also in the Imaging Setup. The Range column displays the detection range of each channel. For internal channels the detection range can be adapted as described above. In addition it is possible to edit the upper and lower border of the detection range by clicking into the range column. Use the arrows or the editing field to precisely adapt the detection range as needed.

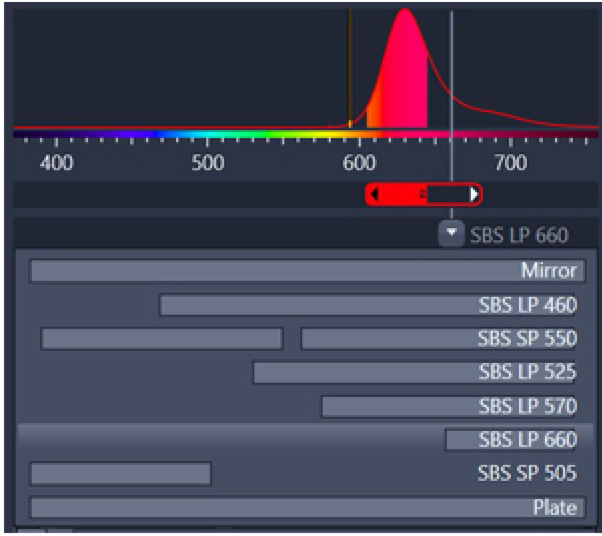


6 **Reflection**

Check the box for imaging in reflection mode. This mode provides an easy tool to get a better understanding of surface structures.

7 **Secondary beam splitters**

The line with the drop down arrow represents the secondary beam splitter (SBS) filter wheel. It allows to direct light to the DCout port where or might be attached. The SBS filter wheel is automatically set to mirror for LSM confocal tracks as typically only internal channels are used in this mode. Mirror directs all emission light to the internal channels. If light should be detected by or as another confocal channel, click the arrow and select a filter from the drop down list. Long pass filters will direct light of longer wavelength to or . Short pass filters will direct light of shorter wavelength to or . The filters can also be selected by moving the line from left to right. The detection range of the internal channels might be affected by using a SBS filter. This is marked by a (partially) empty bar slider for an internal channel.



8 **Icon to select polarization filters**

The emission light can be filtered using polarization filters. The filters are set in track wise acquisition mode for detecting S and P polarized light. With this approach polarizsation anisotropy can be measured. Click onto the icon to select one of the two polarization filters. See *Obtaining an Anisotropy Image [▸ 915]*for further instructions.

9 **Main dichroic and laser line icon for visible lasers (445, 488, 514, 543, 561, 594, 639 nm)**

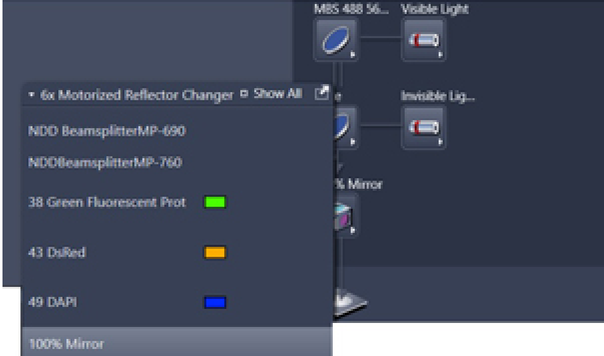
The two icons are one for the main beam splitter and one for the laser line which is coupled to the port facing the corresponding main dichroic filter wheel. When selecting a laser line either in this tool window or otherwise (see Lasers Tool or *Channels Tool [▸ 702]*) the matching main dichroic filter is set automatically. The filter changes again upon selection of an additional laser line or a different laser line if necessary. Deactivation of a line does not change the main dichroic filter. In case no matching dichroic is available the 80/20 dichroic is selected. You can change the dichroic to a self-chosen setting if needed. Note that only after chosing an additional line or unselecting and reselecting a different (combination of) line(s) the filter will be changed again automatically.

10 **Main dichroic and laser line icon for in-visible lasers (405 nm, Multiphoton Laser)**

The two icons are one for the main beam splitter and one for the laser line which is coupled to the port facing the corresponding main dichroic filter wheel. When selecting a laser line either in this tool window or otherwise (see Lasers Tool or *Channels Tool [▸ 702]*) then the matching main dichroic filter is automatically set. The filter changes again upon selection of an additional laser line or a different laser line. Deactivation of a line does not change the main dichroic filter. In case no matching dichroic is available the 80/20 dichroic is selected. You can change the dichroic to a self-chosen setting if needed. Note that only after chosing an additional line or reselecting a different (combination of) line(s) the filter will be changed again automatically. Make sure to deselect all lines first before selecting a new (combination of) laser (lines).

11 **Reflector Turret icon**

When LSM 980 is attached to the rear port of Axio Observer it can be necessary to control the position of the reflector turret of the stand when acquiring a LSM image. For multiphoton systems two positions of the reflector turret are equipped with specific dichroics to reflect the emission signal to NDD channels in reflection. When using NDD tracks (see *NDD (Non Descanned Detector) [▸ 973]*), select the matching dichroic for NDD detection.



14.1.3.2 Airyscan SR

Chose Airyscan SR as acquisition track for superresolution imaging with highest image quality. The following channel is available for Airyscan SR acquisition:

- ChA = GaAsP PMT (optional, part of a configuration with)

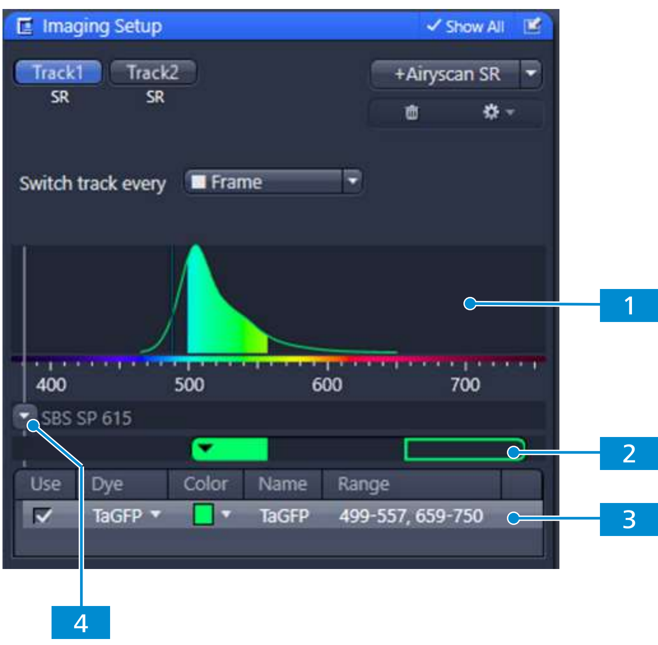


Fig. 104: Imaging Setup, Airyscan SR

For Airyscan SR tracks the SBS filter wheel is automatically set to plate to direct all emission light to .

All other controls are identical to LSM confocal tracks.

Airyscan SR tracks can be combined in a multitrack acquisition with LSM confocal tracks.

1 Spectral display

Shows the emission spectrum of the selected dye assigned to and the laser lines used for excitation.

2 Secondary beam splitters

The line with the drop down arrow represents the secondary beam splitter (SBS) filter wheel. It allows to direct light to the DCout port to or . The SBS filter wheel is automatically set to plate for Airyscan SR tracks. If Airyscan SR tracks should be combined with LSM confocal tracks, the SBS filteres can be used to split the light between the internal channels and Airyscan.

Click the arrow and select a filter from the drop down list. Long pass filters will direct light of longer wavelength to . Short pass filters will direct light of shorter wavelength to Airyscan. The filters can also be selected by moving the line from left to right. The detection range of might be affected by using a SBS filter. This is marked by a (partially) empty bar representing the detection range.

3 Emission filter for Airyscan

Chose a matching emission filter from the drop down list which opens up when clicking the arrow on the left hand side of the bar.

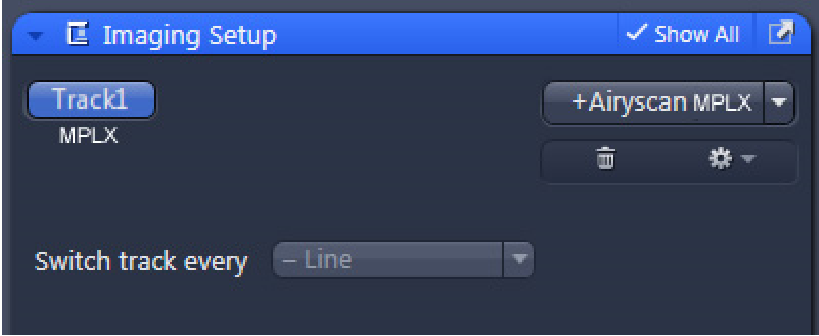
4 **Channel selection area**

This panel displays the per default activated Airyscan channel. Assign a dye to the channel by clicking the Dye drop down arrow and selecting a dye from the Add Dye or Contrasting Method Tool Window. Assign a color to the channel by clicking the arrow in the Color column and selecting a color from the LUT. The color can also be changed in the Channels tool. The name changes to the name of the dye if a dye is selected for this channel. The name can be changed in the Channels tool and then shows up also in the Imaging Setup. The Range column displays the detection range of the Airyscan channel. It is dependent on the selected emission filter.

14.1.3.3 Airyscan MPLX

Multiplex modes allow a faster image acquisition with Airyscan 2 detectors. The acquisition is parallelized in the Y-direction, which allows to process full SR or confocal resolution though not every line in Y was scanned during acquisition. The Airyscan 2 detector has nonetheless acquired data for all lines in the image.

Airyscan MPLX can be selected in the drop down list of the **Imaging Setup**.



For information on how to acquire LSM images with Airyscan multiplex modes, see *Acquiring LSM 900 images with Airyscan 2 multiplex modes [▸ 76]* or *Acquiring LSM 980 images with Airyscan 2 multiplex modes [▸ 79]*.

14.1.3.4 LSM Lambda

Chose LSM Lambda as acquisition track for spectral imaging with subsequent dye unmixing (emission fingerprinting). The mode allows to separate strongly overlapping emission signals.

The following channels are available for LSM Lambda acquisition:

- Ch1 = Multialkaline PMT (part of a 6 or 32 channel configuration)
- ChS = GaAsP PMT (part of a 6 or 32 channel configuration)
- Ch2 = Multialkaline PMT (part of a 6 or 32 channel configuration)
- Ch2 = GaAsP PMT (part of a 3 channel configuration)

Fig. 105: Imaging Setup, LSM Lambda

Info

Lambda tracks cannot be used in a multitrack acquisition.

1 Dye selection panel

Add the emissions spectra of the dyes to be unmixed to the spectral display panel. When clicking **+** the **Add Dye or Contrasting Method** selection panel appears. Chose the corresponding dye and repeat the step for any further dye within your sample. The emission spectra are displayed in the spectral display panel.

2 Spectral display panel

The emission spectra of all selected dyes are displayed in this region to get a better overview over the needed detection range and possibly the necessary spectral resolution.

3 Resolution

Chose the spectral resolution in the drop down list. The **Number of Scans** which will be performed for the selected resolution is displayed on the right side. Depending on the scan head configuration the acquisition of the emission fingerprint image is either done in parallel with one illumination of the sample (32 channel configuration with 8,9 nm or lower spectral resolution) or in several illumination steps (32 channel configuration with 4,3 nm or higher spectral resolution; 6 and 3 channel configuration).

4 Detector selection panel

Activate the necessary detectors for the spectral acquisition. If the spectra do not reach out into the far red or the lower blue range, ChS (or Ch2) suffice for image acquisition.

14.1.3.5 LSM Online Fingerprinting

Chose LSM Online Fingerprinting as acquisition track for spectral imaging with automatic subsequent dye unmixing (Emission Fingerprinting). The mode allows to separate strongly overlapping emission signals with final reduced data volume as no raw data are stored. The mode speeds up data generation in case the parameters for unmixing are known.

The channel for Online Fingerprinting is selected automatically.

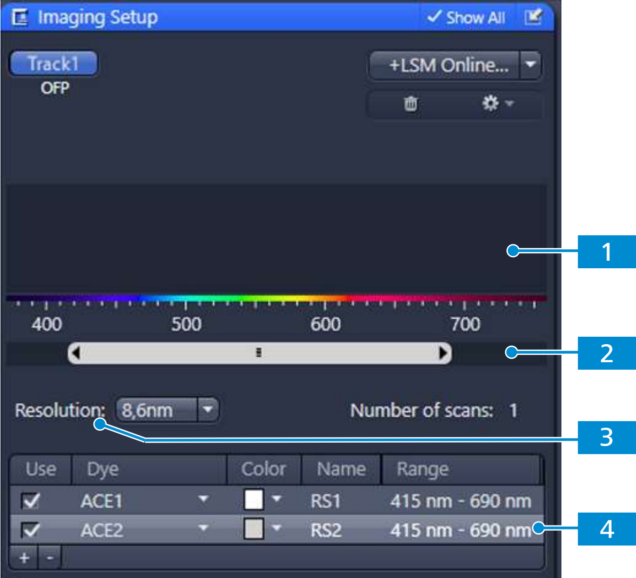


Fig. 106: Imaging Setup, LSM Online Fingerprinting

Info

Online Fingerprinting tracks cannot be used in a multitrack acquisition.

1 Spectral display panel

This area displays the emission spectra of the selected dyes.

2 Spectral range slider

Define the spectral width for the collection of the fluorescent signals by moving, expanding or shrinking the bar slider using the left mouse button.

3 Resolution

Chose the spectral resolution in the drop down list. The Number of scans which will be performed for the selected resolution is displayed on the right side. Depending on the scan head configuration the acquisition of the emission fingerprint image is either done in parallel with one illumination of the sample (32 channel configuration with 8,9 nm or lower spectral resolution) or in several illumination steps (32 channel configuration with 4,3 nm or higher spectral resolution; 6 and 3 channel configuration).

4 Dye selection

Select one or more dyes from the unmixing spectra database. Online Fingerprinting requires pre-defined spectra which will be used for the automatic subsequent unmixing process. The settings for Online Fingerprinting (resolution, spectral width) should ideally match the acquisition parameters for the unmixing spectra.

14.1.3.6 NDD (Non Descanned Detector)

Chose NDD as acquisition track for multiphoton imaging. Using non descanned detectors for imaging when exciting with a multiphoton laser provides a significantly better image quality for deep tissue imaging.

Info

Filters in front of NDDs have to be inserted/changed manually. The indication of changed filters to ZEN software needs to be done with the MTB software. It is possible to do this when ZEN is running by clicking on **Apply** after changing the filters as needed.

The following channels are available for NDD acquisition (all channels are optional; if non-descanned detectors are not available, NDD tracks cannot be set up; the maximum number of channels in reflection/transmission mode is limited to 5 and dependent on the type of stand used):

- NDD.2 (2 channels) in reflection mode (for all stands)
- NDD.2 (2 channels) in transmission mode (for Axio Observer and Axio Examiner)
- NDD.2 single channel as channel 3 or 4 in reflection mode (only in combination with NDD.2; all stands; for Axio Examiner also in transmission mode))
- BiG.2 (2 channels) in reflection mode (for all stands)
- BiG.2 (2 channels) in transmission mode (for Axio Examiner)
- GaAsP NDD 2 channels in reflection mode (for Axio Examiner only, in addition to other channels in reflection mode)

Fig. 107: Imaging Setup, NDD

Info

NDD tracks can be used in multitrack acquisition with other NDD or LSM confocal tracks.NDD tracks can be used in multitrack acquisition with other NDD or LSM confocal tracks. When the tracks are switched between NDD and other modes during an experiment, the system shows a safety warning which, however, does not impair data acquisition but is only commenting on the intermediate state where the correct safety measures kick in.

1 **Display of detection range as colored bar for NDDs positioned in reflection mode**

This area displays the detection range of all selected channels. Only detectors which collect the fluorescent signal reflected from the sample are represented in this line.

2 **Spectral display area**

The emission spectra of dyes allocated to active channels are displayed.

3 **Display of detection range as colored bar for NDDs positioned in transmission mode**

This area displays the detection range of all selected channels. Only detectors which collect the fluorescent signal transmitted through the sample are represented in this line.

4 **Channel selection area**

This panel lists all available detection channels for NDD mode. Select / unselect checkbox Use for activation / deactivation of individual channels. Assign a dye to a channel by clicking the Dye drop down arrow and selecting a dye from the Add Dye or Contrasting Method Tool Window. Assig a color to the channel by clicking the arrow in the Color column and selecting a color from the LUT. The color can also be changed in the Channels tool. The name column lists the names of the detection channels. The name changes to the name of the dye if a dye is selected for this channel. The name can be changed in the Channels tool and then shows up also in the Imaging Setup. The Range column displays the detection range of each channel which depends on the emission filter placed in front of the channel.

14.1.3.7 LSM FCS

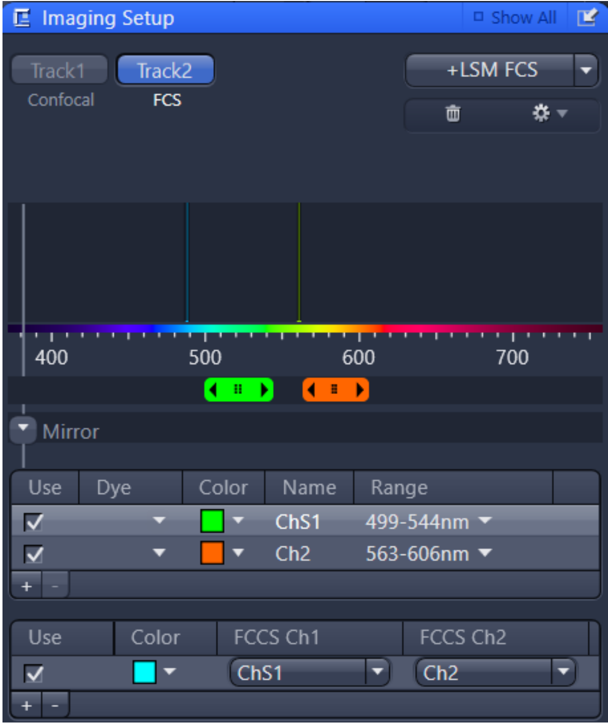


Fig. 108: Imaging setup. LSM FCS

The **Imaging Setup** tool allows to chose LSM FCS as acquisition mode for FCS/FCCS measurements. This track lists all available detectors which can be used for FCS/FCCS measurements. The number of detectors changes with the configuration of the system. A maximum of seven (32+2 ch configuration), 5 (6+2 ch configuration) or 2 channels (2+1 ch configuration) can be selected for FCS/FCCS. An additional 2 detectors are listed independent of the configuration in case BiG.2 is mounted onto the DCout port of the scan head.

The following channels are available:

- ChS1 to ChS4 = GaAsP PMT (part of a 6 or 32 channel configuration)
- Ch2 = Multialkaline PMT (part of a 6 or 32 channel configuration)
- Ch2 = GaAsP PMT (part of a 3 channel configuration)
- GaAsP 1 and GaAsP 2 = GaAsP PMT (optional, detection module attached to DCout port)

Activate one or more detectors as needed for the experiment. In the lower panel these detectors can then also be activated for cross-correlation (FCCS) data analysis.

14.1.4 System Maintenance

Use the **System Maintenance and Calibration** dialog within the **Tools** menu to check on essential parameters of the LSM.

1. Open **Tools** | **System Maintenance and Calibration…**
2. Use the **Default** mode for a user's routine.
3. Perform the following maintenance procedures as described in the safety and operating manual:
   - Scanner calibration
   - Pinhole to MBS calibration
4. Follow the instructions of the wizard.

→ In case the test does not pass you might want to repeat the procedure. If the problem persists you need to check with your local ZEISS service engineer.

The following additional tests provide information on the quality of the system:

- The scanfield test provides information on the quality of the scanner calibration. If this quality repeatedly fails although the scanners have been calibrated, it might be necessary to exchange the scanners. Contact your local ZEISS service engineer.
- The sharpess test provides information on the resolution contrast. This parameter is dependent on the overall alignment of the system and the quality of the detectors. If detectors deteriorate then this test failing might be an indication for a necessary exchange of a detector.

14.1.4.1 Collimators Adjustment

The position of the collimator(s) for lasers coupled to the invis port can be adjusted if needed. The position is dependent on the wavelength and the objective used. The collimator position is a function of the system and the position is not part of an experiment and hence cannot be reloaded with an experiment or re-used with an image.

For tunable Multiphoton lasers it might be necessary to adjust the collimator for a given wavelength and objective as the laser is set up together with the LSM first time at the customers.

Use the following parameters to adjust the collimator.

Parameter	Description
Collimator	Chose the collimator you want to adjust. In case no Multiphoton laser lines are available only the collimator for 405 nm laser is accessible.
Position	Use the slider or the editing box to change the position of the collimator. Check the image of a continuously scanned sample (i.e. LSM calibration objective) for highest intensity. In case you want to align the collimator for optimal overlap with vis lasers, perform for example a reflection line scan with vis laser and focus for highest signal. Then adjust the collimator of the invis laser for highest image intensity. If both reflection images show highest intensity the excitation level matches.
Store Current Pos	Stores the current position as the default position for the selected collimator.
Move to Stored Pos	Moves the position of the selected collimator to a stored value for the given wavelength and objective. Stored positions are automatically set when an objective is changed or the laser is tuned (Multiphoton laser). The current non stored settings are kept for an experiment if the laser is not tuned or the objective is not changed.

14.1.4.2 Pinhole Adjustment

The position of the pinhole (or rather the laser beam in X-Y-coordinates) in relation to the detector makes a major contribution to image optimization.

The pinholes have already been adjusted at the factory. These settings are taken over for active operation when a standard configuration is loaded.

If you want to create a setting that differs from the standard configurations, use the following parameters.

Parameter	Description
Diameter [µm] slider	Changes the diameter of the pinhole
X Position [Vis]	Changes the X position of the excitation beam of all vis lasers in relation to the pinhole
Y Position [Vis]	Changes the Y position of the excitation beam of all vis lasers in relation to the pinhole
X Position [Invis]	Changes the X position of the excitation beam of all invis lasers in relation to the pinhole
Y Position [Invis]	Changes the Y position of the excitation beam of all invis lasers in relation to the pinhole
Store Current Pos	Stores the current position as the default position for the given lasers and main dichroic filter
Moved to Stored Pos	Resets the pinhole position to the stored value

14.1.4.3 Periscope Adjustment

Multiphoton lasers are steered into the scan head of the LSM via a free beam coupling. The components of the coupling kit ensure that the laser beam is enclosed within metal pipes. The coupling components include a periscope which lifts the free beam up to the height it needs to have to hit the coupling port of the scan head. The positioning of a freely coupled laser beam is subject to temperature changes, potentially mechanical influences and the laser itself. When tuning the laser, it very slightly changes its position. When working with two laser beams the precise overlay of the two beams can be critical for the experiment. Hence the “Adjust Periscopes” tool provides access to the motorized steering mirrors within the periscope to adjust for possible mismatches between the two laser beams.

The periscope contains two sets of mirrors for each beam. One set has more influence onto the homogeneity of the illumination, the other set is rather adjusted for a precise overlay between the two beams. For a good coupling both the lateral position as well es the angle of the beam needs to be adjusted to have the laser travel along the center of the optical axis.

The best reference to achieve this is an image of a structured and fluorescent sample taken with the 488 nm laser. Such a sample can also be purchased at ZEISS.

The following controls are available:

Parameter	Description
Reference Image	A drop-down list opens listing all open images with the last one acquired on top of the list. The selected image appears as overlay image in the continuous image tab.
Opacity	Opacity relates to the reference image display in the continuous image tab. 100% does only show the reference image, 0% does only show the continuously acquired image.
On	Switches the overlay image on/off (set opacity/0% opacity) for quick cross check on overlaid structures.

Parameter	Description
NLO 690-1300 nm	Click to select the periscope mirrors for the tuneable line of the Multiphoton laser.
NLO 1045 nm	Click to select the periscope mirrors for the fixed line of the Multiphoton laser.
Arrow button for various directions	Depending on the selection of the laser line, the arrows move the beam and hence the continuously acquired image taken with the NLO laser in the indicated direction. The arrows allow fine and course movement steps. The number of steps is displayed next to the arrow buttons.
Move to zero	A current absolute position of each mirror pair can be defined as position zero. This resets the number of moved steps to zero.
Set position zero	Moves the mirrors back to the zero position. The position of the mirrors present at each start of the system is taken as initial zero.

14.1.4.3.1 Correcting inhomogeneous illumination

Prerequisite

- ✓ Sample with homogeneous fluorescence is placed in the sample holder (the samples fluorescence must be excitable by any NLO line).
- ✓ The preferred objective for the alignment for Axio Observer: 10x; for Axio Examiner: 20 x 1.0 .
- ✓ The sample is in the field of view and focused.
- ✓ The tuneable Multiphoton laser line is tuned to the desired wavelength.
- ✓ The acquisition parameters are set to have the maximum field of view and imaging speed of 9 or 10 to quickly see the changes in the image when clicking the alignment arrows.
- ✓ Imaging Setup is configured using one internal detection channel at relevant spectral range with open pinhole.
- ✓ Multiphoton laser is activated for the single channel acquisition track.

1. Open **Tools > System Maintenance and Calibration**.
2. Select the tool **Adjust Periscopes**.
3. Select laser line of which the periscope mirrors should be controlled to check/align for homogeneous illumination.
4. Start a **Continuous** scan.
5. Set laser power and detector gain for optimal imaging with just the first pixels in saturation (check with range indicator LUT).
6. In case the brightest area is not centered use arrow keys to center the brightest area.
7. Use also a profile line for better visualization of the intensity distribution.
   → The motor steps of each mirror are counted to easily move back to the original value.
8. Stop continuous scan when illumination is optimized.
9. The final mirror positions can be set as new zero.
   → This position will be constant unless the mirrors are moved again.

14.1.4.3.2 Correcting overlay of both NLO laser lines

Prerequisite

- ✓ Sample with homogeneous fluorescence and structures with high contrast is placed in the sample holder (the samples fluorescence must be excitable by any NLO line).
- ✓ Chose the objective relevant for the application.
- ✓ Higher NA allows for higher precision when checking and correcting the overlay.
- ✓ The sample with its structured part is in the field of view and focused.
- ✓ A single snap image using preferably the 488 nm laser is acquired as reference image with good visibility of the structures.
- ✓ The tuneable NLO laser line is tuned to the desired wavelength.
- ✓ Imaging Setup is configured using one internal detection channel at relevant spectral range with open pinhole.
- ✓ The acquisition zoom and the stage position are kept constant and are identical to the acquisition of the reference image.

1. Open **Tools > System Maintenance and Calibration**.
2. Select the tool **Adjust Periscopes**.
3. Select the just acquired single image as reference image.
4. Select laser line of which the periscope mirrors should be controlled to check/align for image overlay.
5. Activate this laser for acquisition in the Channels tool.
6. Start a **Continuous** scan.
7. Set laser power and detector gain for optimal imaging.
8. Set opacity of the reference image such that the structures of the reference image and the continuously scanned NLO image are visible.
9. Use the arrow keys for overlay to overlay the structures of the continuously scanned image with the reference image.
10. It might be necessary to use the overlay and illumination mirrors alternately to achieve the optimum result.
11. The motor steps of each mirror are counted to easily move back to the original value.
12. The reference image can be quickly switched on/off for quick alternating display of the structures.
13. The final mirror positions can be set as new zero. These positions will be constant unless the mirrors are moved again.
14. Repeat step 4 to 12 for the other NLO laser line.

Info

If the alignment of the Multiphoton laser is off and only part of the beam or no beam is visible in the objective plane do not attempt to align using this tool. The tool is designed to correct for minor mismatches occurring when the laser is tuned or small influences on minor temperature fluctuations. It is assumed that the system is set up in a temperature-controlled room without major air flow as is described in the system setup requirements.

14.1.4.4 Airyscan Detector Adjustment

14.1.4.4.1 Introduction

The tool provides controls for the automatic alignment of the Airyscan detector during system operation. The status of the current adjustment is indicated in the status bar of ZEN whenever the Airyscan detector is in use in Airysan SR or Airyscan Multiplex tracks.

The tool can be opened via the **System Maintenance and Calibration** dialog located in the **Tools** menu or by clicking the arrow next to the icon of the detector in the status bar.

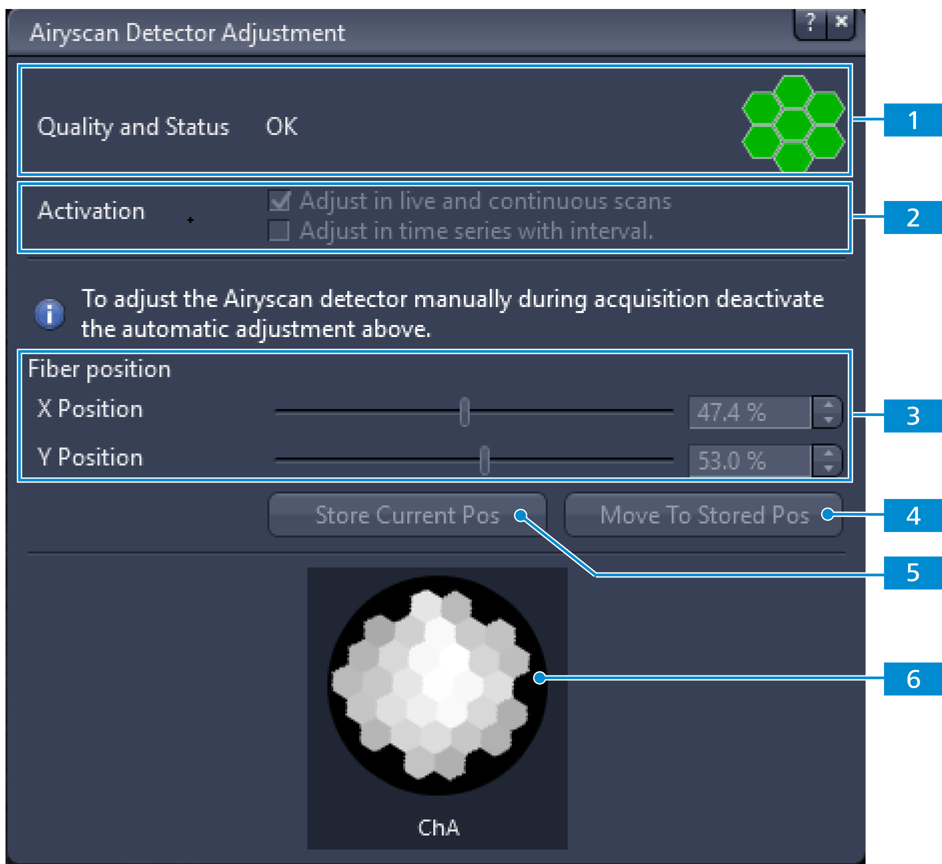


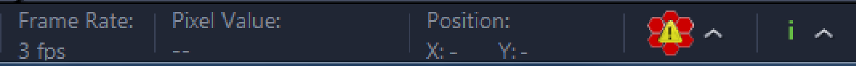
Fig. 109: Airyscan Detector Adjustment tool

No.	Parameter	Description
1	**Quality and Status**	Shows the adjustment status.
	-	No signal available
	-	Not enough light or too much

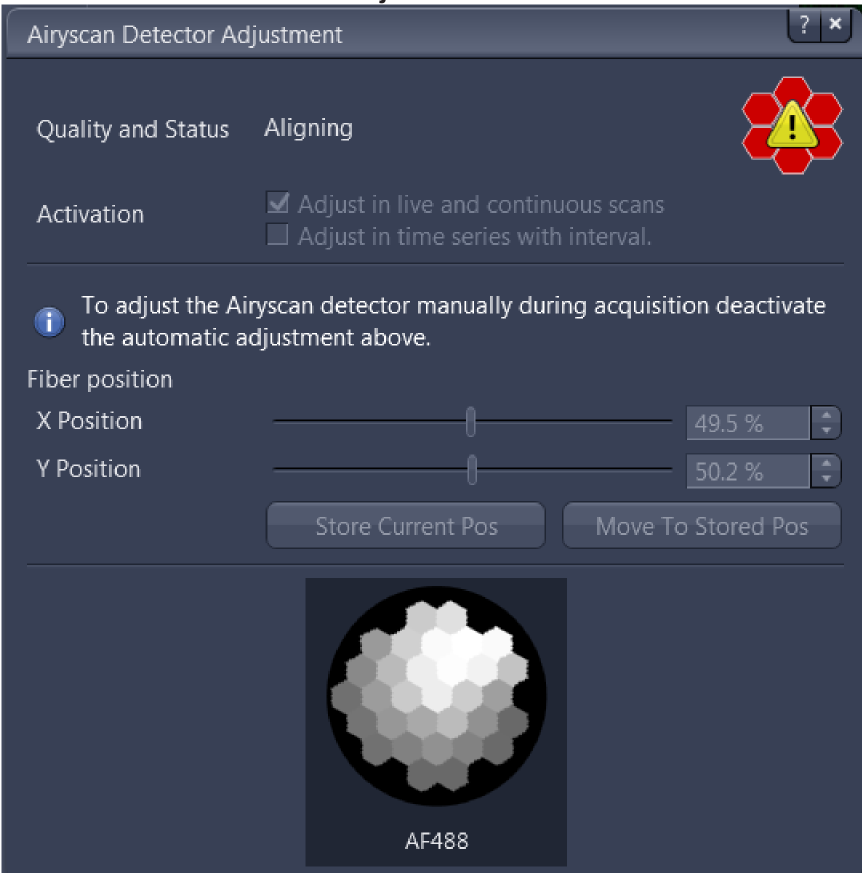
No.	Parameter	Description
	-	Alignment ongoing
	-	Alignment done and ok
	- **Manual Mode:**	The adjustment can be done using the sliders under **Fiber position**.
	- **Automatic Mode:**	The adjustment is performed as defined in **Activation**.
2	**Activation**	Activate the alignment during **Live** and **Continuous** mode. Activate the alignment during **Time Series Acquisitions** with **Interval**.
3	**Fiber position**	Use slider to change the adjustment of the fiber in x and y for manual positioning. The slider can only be operated in **Manual Mode**.
4	**Store Current Pos**	Stores the current positions of x and y for further reference.
5	**Move to Stored Pos**	Moves both sliders to the stored position. Going to the stored position is a good start when manually realigning the detector.
6	**Detector View**	Shows the current intensity distribution of the emission signal over the detector elements.

14.1.4.4.2 Adjusting the Airyscan Detector automatically via the status bar

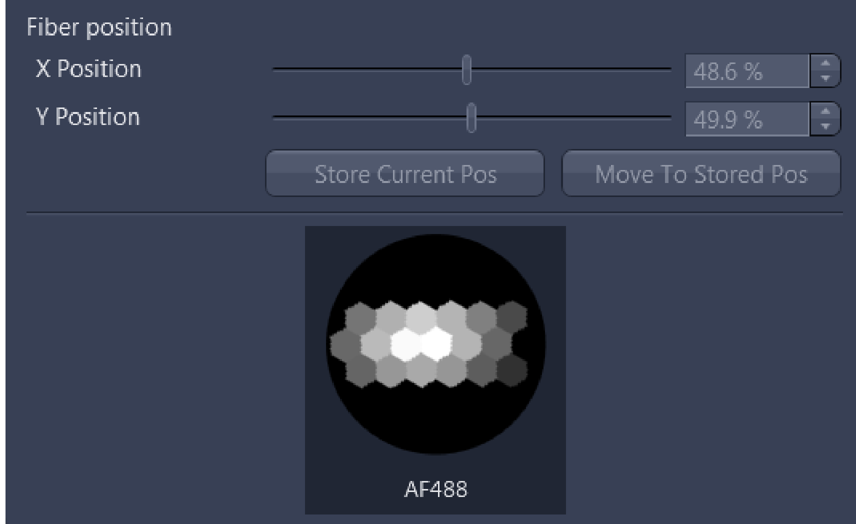
1. To access the detector adjustment, click on the detector symbol at the Status bar. Depending on the selected Airyscan mode, the illumination pattern varies in geometry. A correct adjustment is always symmetrical and centered.



2. Start a live or continuous scan to automatically adjust the Airyscan 2 detector.
3. Activate the checkbox **Adjust in live and continuous scans**. Alternatively, start a time series with intervals and activate **Adjust in time series with interval**.



The pattern of the active Airyscan detection elements might vary depending on the chosen Airyscan detection mode.



14.1.4.4.3 Adjusting the Airyscan Detector automatically via System Maintenance and Calibration

Info

When using 405nm laser in one track and any other laser in the second track, use continuous mode with both tracks active for Airyscan detector adjustment. The alignment for 405nm laser depends on the alignment of the other lasers first. If the tracks are adjusted individually the alignment is mutually overwritten and cannot work properly.

Info

In case the experiment requires image acquisition in multi-track mode with frame wise switching between the tracks and one track using the 405 nm laser for excitation, the following setting needs to be defined to achieve an automatic adjustment of the Airyscan detector during Continuous mode: Keep the main dichroic for the 405 laser identical between all tracks whether the tracks work with the 405 laser or not. Otherwise the switching of the MBS between the tracks does not allow to finalize the adjustment and the system stops with an error message. Do also check the suggestions for track setups which are made by Smart Setup..

Info

When Airyscan is used as detector for LSM confocal track, alignment is not needed. The alignment is set to the stored position.

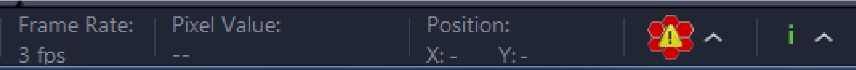
Use manual mode if the alignment takes to long or does not work. Move slider to the stored position first for a good start.

Prerequisite
- ✓ **Airyscan SR** or **Airyscan MPLX** track is set up
- ✓ Icon for Airyscan Adjustment status visible in the status bar

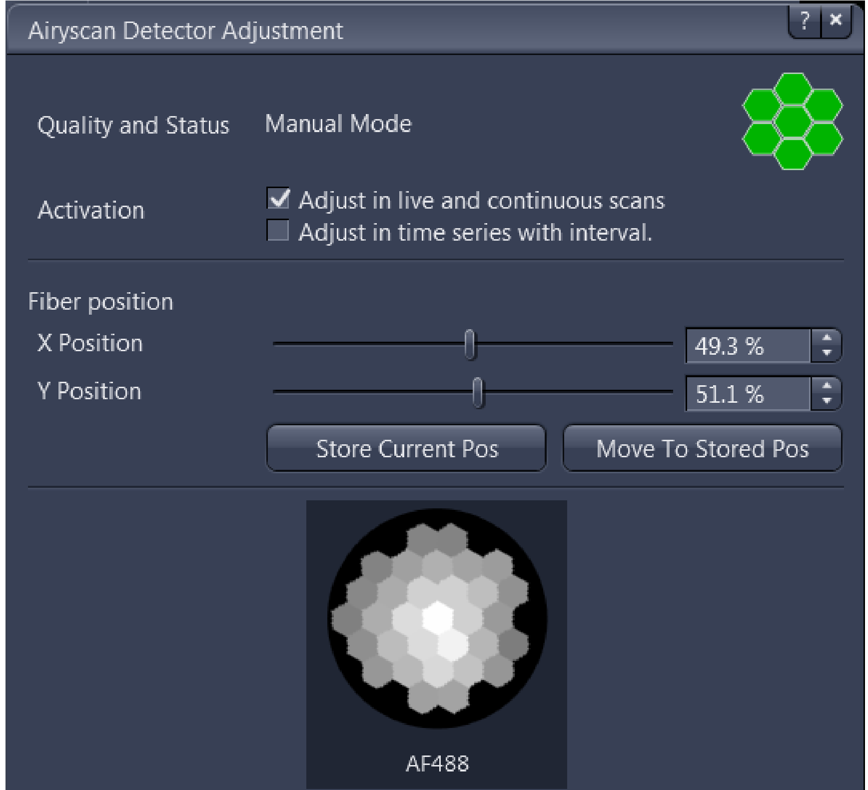
1. Open **Tools > System Maintenance and Calibration ...**
2. Select **Default** as the desired user group .
3. Click on **Continue**.
4. Click on **Airyscan Detector Adjustment**.
   → The **Airyscan Detector Adjustment** dialog appears.
5. Activate **Adjust in live and continuous scans**.
6. Start a **Live** or a **Continuous** scan.
   → When light is reaching the detector, the distribution of the intensity over the detector elements is displayed (detector view icon).
7. Keep scanning and adjust detector gain/laser power in case the light level is to high/low.
8. Increase scanning speed (for **Continuous** mode only) to speed up the alignment procedure.
   → When the light level is ok, the icon shows in red with a yellow warning triangle and the alignment is still ongoing.
   → When the alignment is done and ok the icon turns green and quality and status ore OK; the detector element with the brightest signal is the one in the center/middle line (depending on the imaging mode SR versus MPLX).
   → With stable room conditions according to the specifications (see **Safety and Operation** instructions) there is no need to readjust the detector for every new experiment.

14.1.4.4.4 Adjusting the Airyscan Detector manually

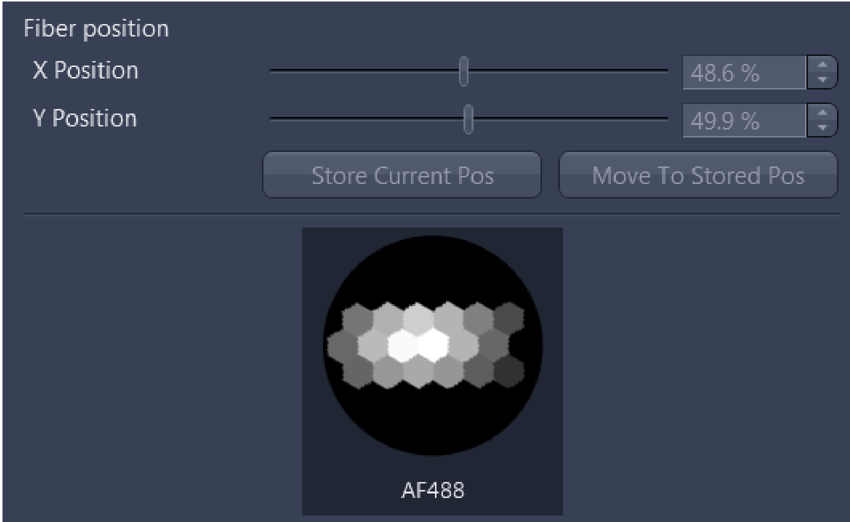
1. Click on the detector symbol at the Status bar. Depending on the selected Airyscan mode, the illumination pattern varies in geometry. A correct adjustment is always symmetrical and centered.



2. Deactivate all check boxes.
3. Move the sliders for the **Fiber position** of X and Y until the detector symbol turns green.
4. Click on **Store Current Pos** to save the adjustment setting.
5. Click on **Move To Stored Pos** to override the position found by the check boxes and will keep this position if live or continuous scan is off.



The pattern of the active Airyscan detection elements might vary depending on the chosen Airyscan detection mode.



14.1.4.5 Adjusting Readback Signals

The following function adjusts the readback signals of the scanner when a spline scan is performed (see *Parameters for LSM Imaging Modes [▶ 688]*)

To adjust the readback signals proceed as follows:

1. Set image acquisition to mimimal zoom and a slow speed like 5 or 6.
2. Chose **Acquisition** tab | **Acquisition Mode** tool |**Line** scan mode.
3. Click **Line select >>** to start a continuous scan.
4. Select **LineSelect** tab | **Spline Mode**.
5. Draw a scan line ideally covering a large area of the image.
   → The readback signal curve will show up as a second line.
6. If this readback signal does not match with the drawn line, use the various sliders (**Factor and Position** for X and Y) to achieve maximum overlay between the two lines.
7. For the spline scan itself it might then be necessary to reduce the scan speed and / or reduce the bend of the spline curve to achieve optimal spline scan positioning.

The adjustment of the readback signal should be checked now and again if the overlay of the signal with the spline graphics does no longer sufficiently match.

14.2 ApoTome.2

14.2.1 Introduction

In the following chapters you will learn how to calibrate the **ApoTome** for a two-channel experiment and acquire a two-channel image. This image will be used as a basis for demonstrating the processing options. After this, a Z-Stack image will be acquired and processed with the help of ApoTome deconvolution.

Phase calibration, if it has not yet been performed, is carried out from the **Locate** tab, while the other steps are all performed from the **Acquisition** tab.

Grid Focus Calibration is an important step. It is best to perform this using the sample that you will want to acquire later, to guarantee identical optical conditions. If your sample is prone to significant bleaching, you can also use the calibration slide provided.

Background information on the ApoTome can be found here:

- *Principle of imaging using fringe projection [▶ 985]*
- *Optimum acquisition conditions [▶ 991]*
- List of recommended objectives

14.2.2 Principle of imaging using fringe projection

The optics of a microscope are optimized for analyzing very thin samples. For a cover-slip-corrected objective, all optical calculations are performed for very thin objects that lie directly beneath the cover slip. All cover-slip-corrected objectives from ZEISS are optimized for this particular usage, and exhibit an optimum point spread function (PSF) for the wavelengths for which the corresponding objective has been specified.

In biological applications, however, the vast majority of specimens used do not satisfy these optimum requirements. Sometimes thicker biological tissue slices are used, e.g. to analyze cells in the tissue using specific fluorescent markers.

In such cases, during microscopic analysis, and particularly during documentation, the set focal plane is hidden by parts of the image that originate from above and below the actual focal plane. As a result the image appears "faded", the contrast is reduced, and the background becomes bright. In extreme cases important structures and image details may be completely hidden.

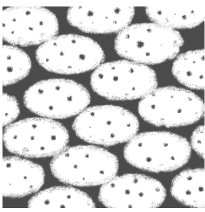


Fig. 110: Input image (schematically)

The above representation of a microscopic image of cell nuclei in tissue shows this effect. A number of methods can be used to prevent or reverse this effect, such as confocal laser scanning microscopy or 3D deconvolution.

Principle of fringe projection

With the **ApoTome 2** the principle of fringe projection has been employed. This technology involves inserting a grid structure with grid lines of a defined width into the plane of the luminous field diaphragm of the reflected light beam path. As the plane of the luminous field diaphragm is conjugated to the focal plane, when you look into the eyepiece you can therefore see the grid, overlaid with the actual sample.

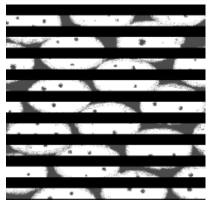
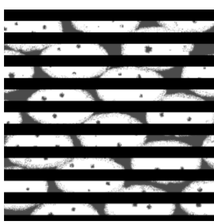


Fig. 111: Grid image

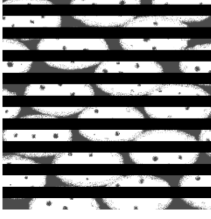
The image of the grid is shown schematically in the figure above. In reality the grid lines are much thinner.

Moving the grid structure

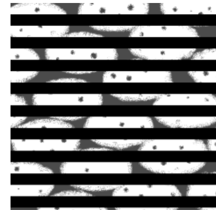
A scanning mechanism in the ApoTome 2 slider is used to move the grid structure in at least three defined steps within the specimen plane. The grid is moved very quickly (in less than 20 ms). A digital image is acquired at each grid position. The next figure shows the movement of the grid schematically:



Position 1



Position 2



Position 3

Optical section

The three raw images are combined online on the PC and displayed as an optical section. This combined resulting image is an optical section through the sample with the following properties:

- The grid structure has been removed from the raw images.
- The parts of the image that are out of focus are no longer visible.
- The sharpness and contrast of the image have been increased.
- The image’s resolution in the axial direction has been increased.

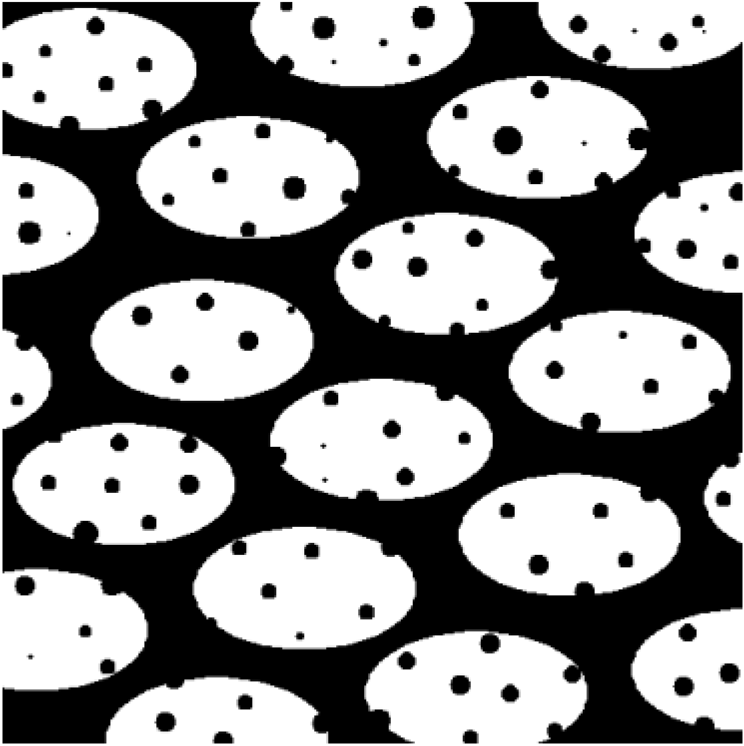


Fig. 112: Output image (schematically)

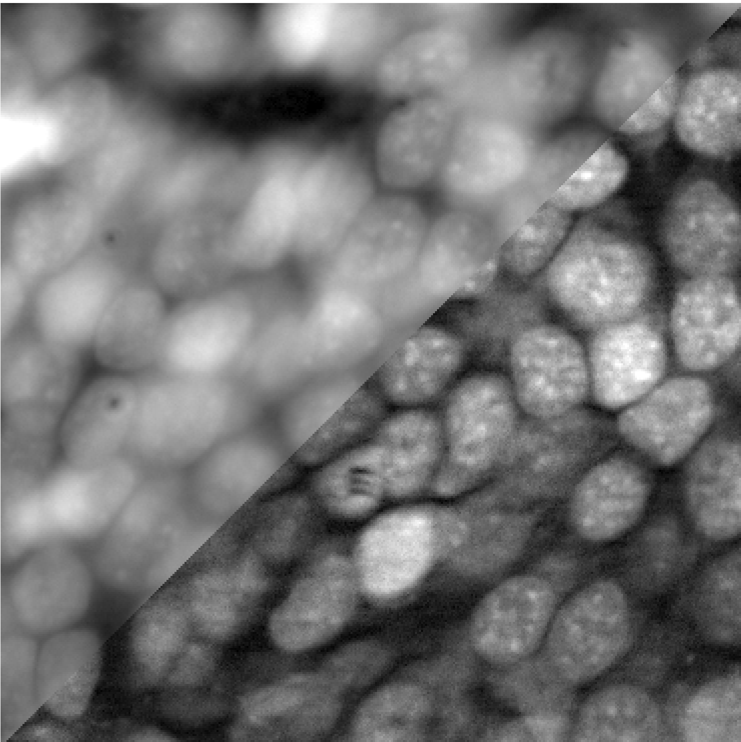


Fig. 113: Sample image: cell nuclei

The figure above shows an application image of cell nuclei (tadpole brain section) in black and white. Above left: conventional fluorescence Below right: optical section

Why is the resulting image an optical section?

Schematic image of the grid

One possible way to explain this is to use the image of the grid in the sample:

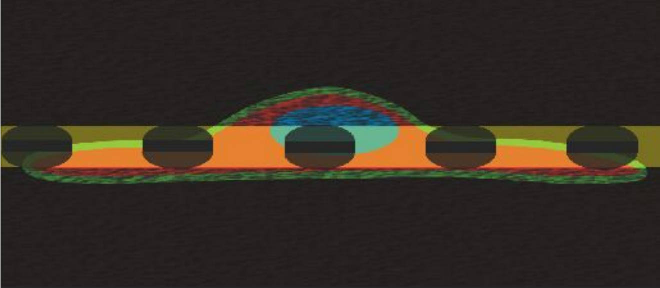


Fig. 114: Grid image (schematically)

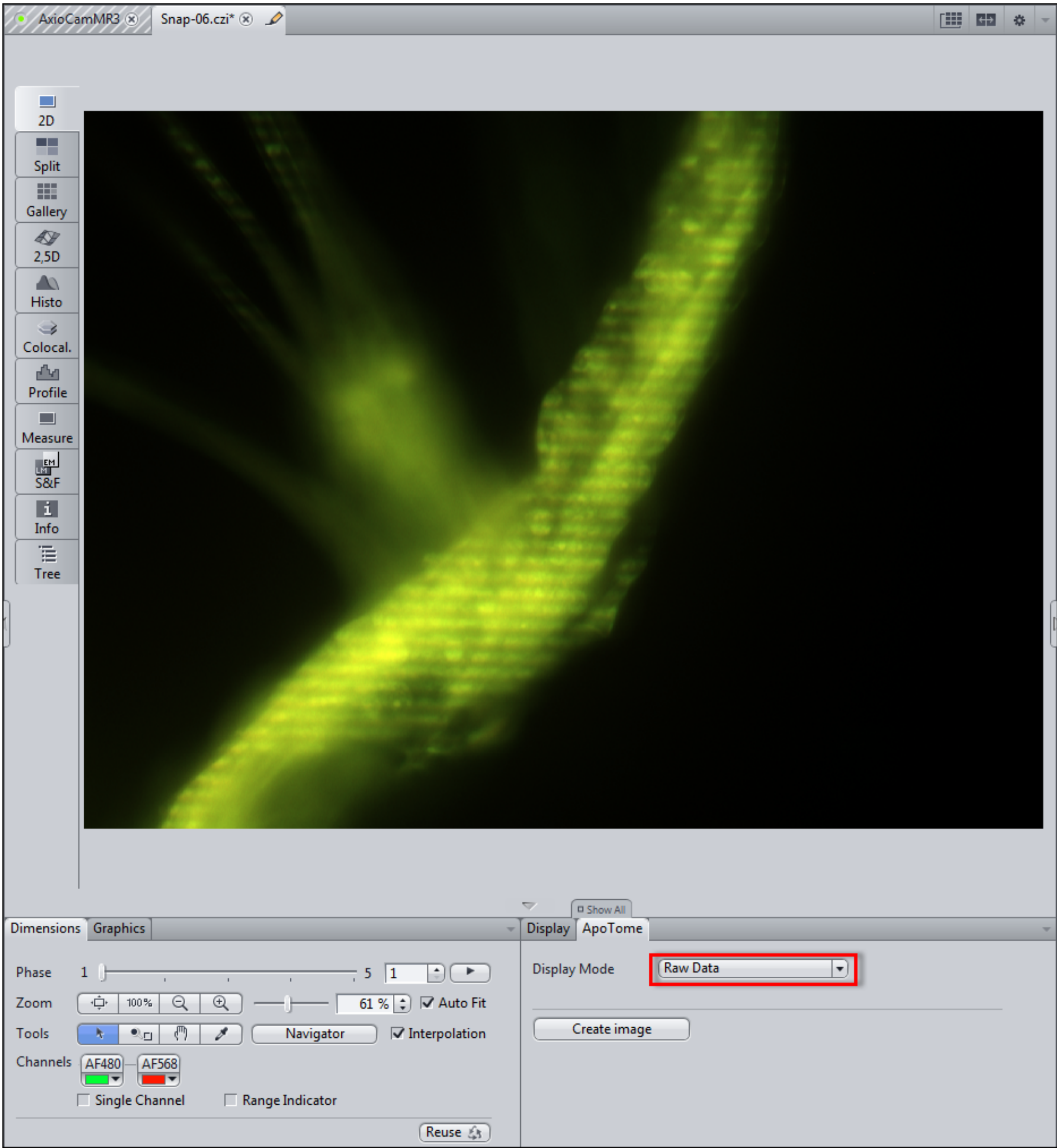
The image of the grid provides the necessary information on the distance of the various specimen structures from the set focal plane (see figure above). Some specimen structures are in focus, while others lie above or below the focal plane, and enter the set focal plane.

The technique of grid projection makes use of the fact that the image of the grid above and below the actual focal plane is blurred, and enters the blurred areas of the specimen. When the grid line is moved, significant brightness differences (= contrast) appear in the focal plane.

Outside the focal plane only minor differences are produced, as the sample and the image of the grid are practically "blurred" together. The brightness differences are detected by the algorithm used to combine the raw images, and are used to remove the parts of the image that are out of focus.

The Raw Data mode

To view the raw data directly switch to the **Raw Data** display mode on the **ApoTome** tab.



You will now see the **Phase** slider on the **Dimensions** tab. Here you can locate the individual grid positions. This view can be useful when looking for errors, e.g. to find out where residual streaks in the processed resulting image originate from.

14.2.3 Optimum acquisition conditions

The following requirements should be met in order to produce optimum ApoTome images:

- **Exposure time of the camera**: this should be set so that approx. 80% of the camera's dynamic range is used. The smaller the dynamic range of the images of the lines, the more noise the combined resulting images will contain.
- **Correct calibration**: Good results can only be achieved if calibration has been performed correctly. Ideally you should use your own sample for calibration. If this does not lead to good results, use the calibration slide provided.
- **Sufficient grid contrast in the sample**: Good section image results can only be achieved if the grid lines in the live image can be clearly identified in all object areas. Under certain circumstances samples with very homogeneous staining throughout may not be suitable for ApoTome images.
- **Avoid vibrations** during acquisition, as any movement of the grid position during acquisition can lead to streak artifacts.
- **Number of phases**: Although 3 grid positions (also called phases) can completely cover the object structures that are in focus and are therefore sufficient for creating optical sections, the results are significantly better when 5 or more phases are acquired. For this reason 5 phases are acquired as standard.
- **Selection of the correct grid frequency**: Under normal circumstances, the automatic grid selection yields the best results. In the case of difficult samples, e.g. if the staining is weak, selecting a different grid manually can lead to better results.
- **Avoid electronic interference**: The ApoTome's scanner unit is equipped with highly precise control. Avoid electrical interference, e.g. leaving cell phones close to the ApoTome, to prevent incorrect positioning of the grid.

14.2.4 List of recommended objectives

The following list gives an overview about the recommended objectives and the compatibility with several microscopes. The links give detailed information about the features of each listed objective.

Overview

- EC Plan-Neofluar (Axio Observer / Axio Zoom.V16) *EC Plan-Neofluar [▸ 992]*
- LCI Plan-Neofluar (Axio Observer / Axio Zoom.V16) *LCI Plan-Neofluar [▸ 992]*
- Plan-Apochromat (Axio Observer / Axio Zoom.V16) *Plan-Apochromat [▸ 992]*
- LD LCIPlan-Apochromat (Axio Observer) *LD LCIPlan-Apochromat [▸ 993]*
- C-Apochromat (Axio Observer) *CApochromat [▸ 993]*
- LD C-Apochromat (Axio Observer) *LD CApochromat [▸ 994]*
- A Plan-Apochromat (Axio Observer) *a Plan-Apochromat [▸ 994]*
- A Plan-Fluar (Axio Observer) *a Plan-Fluar [▸ 994]*

Microscopes

- Objectives for Axio Observer
- Objectives for Axio Zoom.V16

14.2.4.1 EC Plan-Neofluar

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
10x	0.3	Air	2.9 / 31.9	1.7 / 18.2	0.9 / 9.9	OK	OK
20x	0.5	Air	2.4 / 9.2	1.4 / 5.3	0.7 / 2.9	OK	OK
40x	0.75	Air	1.6 / 2.8	0.9 / 1.6	0.5 / 0.9	OK	OK
40x	1.3	Oil	2.5 / 2.2	1.4 / 1.2	0.8 / 0.7	OK	OK
63x	0.95	Air	1.0 / 1.1	0.6 / 0.7	0.4 / 0.4	OK	No
63x	1.25	Oil	1.6 / 1.5	0.9 / 0.9	0.5 / 0.5	OK	OK
100x	1.3	Oil	1.0 / 0.9	0.6 / 0.5	0.4 / 0.3	OK	OK

14.2.4.2 LCI Plan-Neofluar

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
25x	0.8	Oil, water or glycerin	2.9 / 6.6	1.7 / 3.7	0.9 / 2.0	OK	OK
63x	1.3	Water or glyc-erin	1.5 / 1.3	0.9 / 0.7	0.5 / 0.4	OK	OK

14.2.4.3 Plan-Apochromat

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
10x	0.45	Air	4.2 / 20.4	2.4 / 11.5	1.3 / 6.2	OK	OK
20x	0.8	Air	3.2 / 4.9	1.8 / 2.8	1.0 / 1.5	OK	OK

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
40x	0.95	Air	1.6 / 1.7	0.9 / 1.0	0.5 / 0.5	OK	OK
40x	1.3	Oil	2.5 / 2.2	1.4 / 1.2	0.8 / 0.7	OK	OK
40x	1.4	Oil	2.4 / 1.8	1.4 / 1.0	0.7 / 0.6	OK	OK
63x	1.4	Oil	1.6 / 1.2	0.9 / 0.7	0.5 / 0.4	OK	OK
100x	1.4	Oil	1.0 / 0.8	0.6 / 0.5	0.4 / 0.3	OK	OK

14.2.4.4 LD LCIPlan-Apochromat

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
25x	0.8	Oil, water or glycerin	2.9 / 6.5	1.7 / 3.8	0.9 / 2.0	OK	OK

14.2.4.5 CApochromat

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
10x	0.45	Water	4.2 / 20.2	2.4 / 11.7	1.3 / 6.1	OK	OK
40x	1.2	Water	2.1 / 1.9	1.3 / 1.1	0.7 / 0.6	OK	OK
63x	1.2	Water	1.4 / 1.3	0.8 / 0.7	0.5 / 0.4	OK	OK

14.2.4.6 LD CApochromat

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
40x	1.1	Water	2.1 / 2.3	1.3 / 1.4	0.7 / 0.7	OK	OK

14.2.4.7 a Plan-Apochromat

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
63x	1.46	Oil	1.5 / 1.0	0.9 / 0..6	0.5 / 0.3	OK	OK
100x	1.46	Oil	1.0 / 0.7	0.6 / 0.4	0.3 / 0.2	OK	No

14.2.4.8 a Plan-Fluar

V	NA	Immersion	Grid / Section Thickness @ 490nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
			Highgrid	Middlegrid	Lowgrid		
100x	1.45	Oil	1.0 / 0.7	0.6 / 0.4	0.3 / 0.2	No	No

14.2.5 Preparation: Phase calibration

Before you can use the **ApoTome** for your experiments, the optimum angle of deflection of the scanner unit must be set on the **ApoTome 2**. This fine adjustment of the scanner unit only has to be performed once after the system has been set up. The mirror slide and special reflected light reflector cube, both of which are supplied with the ApoTome 2, are used for this purpose.

Calibration only needs to be performed for one grid and one objective. It is advisable to calibrate the grid for the low magnification range (grid marked with an "L" for "Low magnification") using a 20x objective.

The positioning of the camera is also optimized in the dialog for phase calibration. To achieve optimum performance, the camera horizontal should be aligned parallel to the ApoTome 2 grid lines with as much precision as possible.

The calibration process is supported by a wizard. Start the function by selecting the ApoTome Phase Wizard function from the Acquisition menu.

The wizard guides you through the calibration process in 5 steps. Follow the instructions in the text field of the wizard.

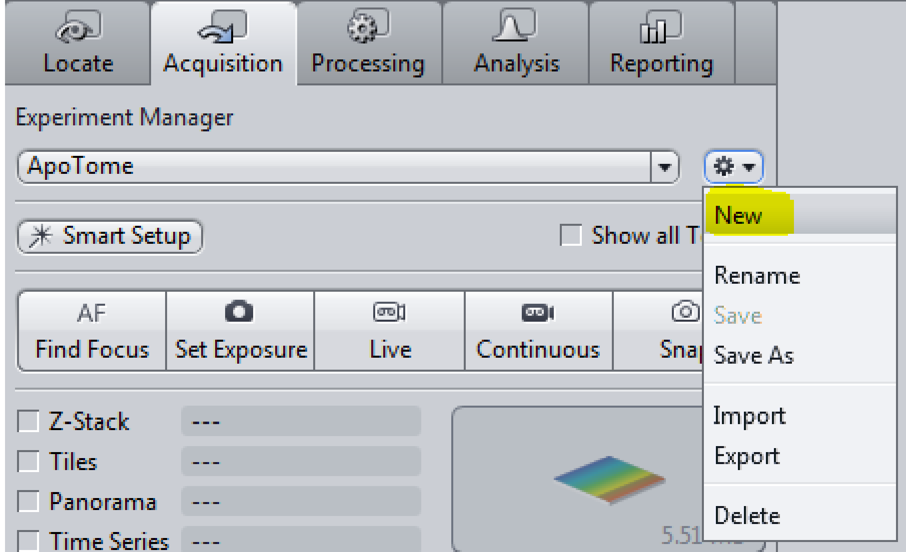
For phase calibration you will need the mirror slide provided and the calibration filter (424930-9902-000). The filter is designed for use with white light sources such as the **HXP120C** and cannot be used with the LED light source **Colibri**. If your ApoTome system was ordered and supplied exclusively with Colibri, a suitable calibration filter (424930-9000-000) has already been provided. If Colibri has been retrofitted and the calibration filter is not available, it is not possible to perform phase calibration. In this case please contact your ZEISS sales representative.

14.2.6 Step 1: Define channels using Smart Setup

Aim

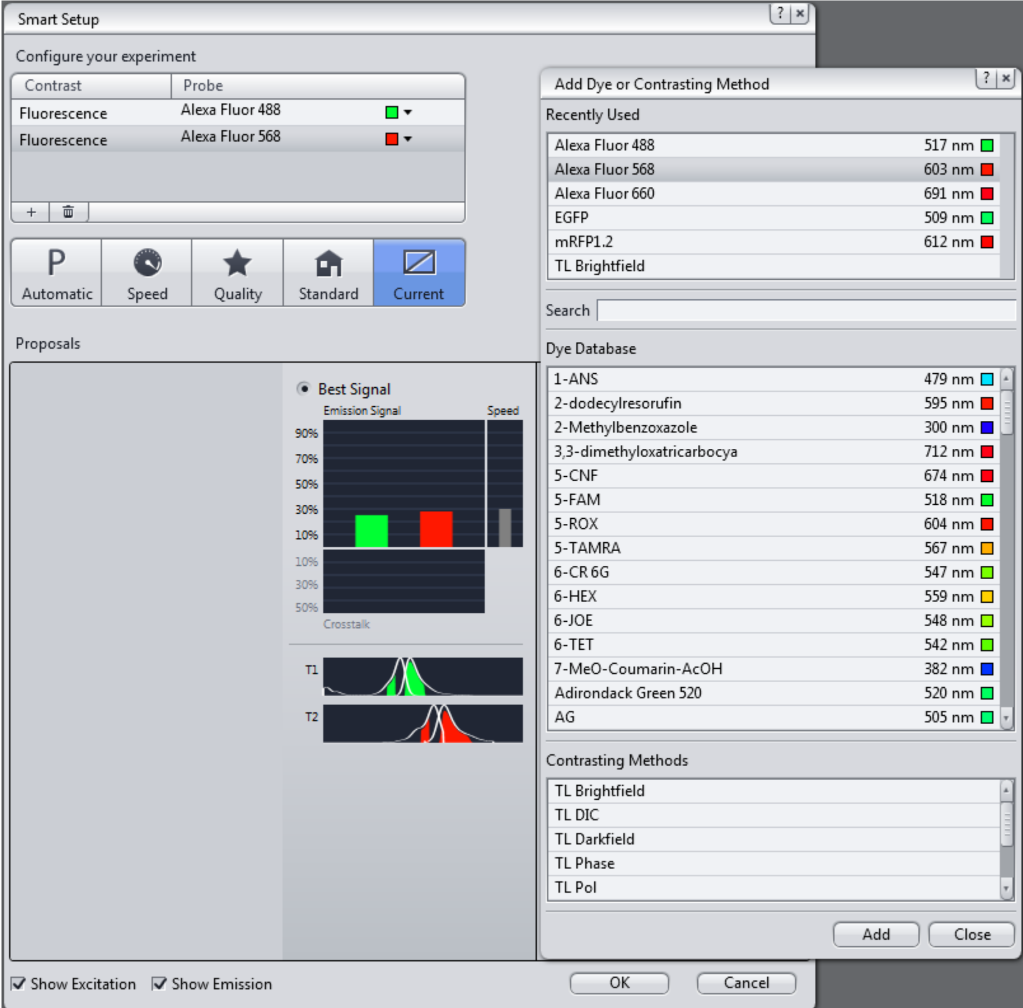
In this step we will set up a two-channel experiment. To do this, we will use the Smart Setup function. The ApoTome is in the first click-stop position, i.e. in the empty position without a grid.

1. Place your sample onto the microscope stage, localize it with the help of the functions on the **Locate** tab and bring it into focus.
2. Now go to the **Acquisition** tab and create a new experiment in the **Experiment Manager**:



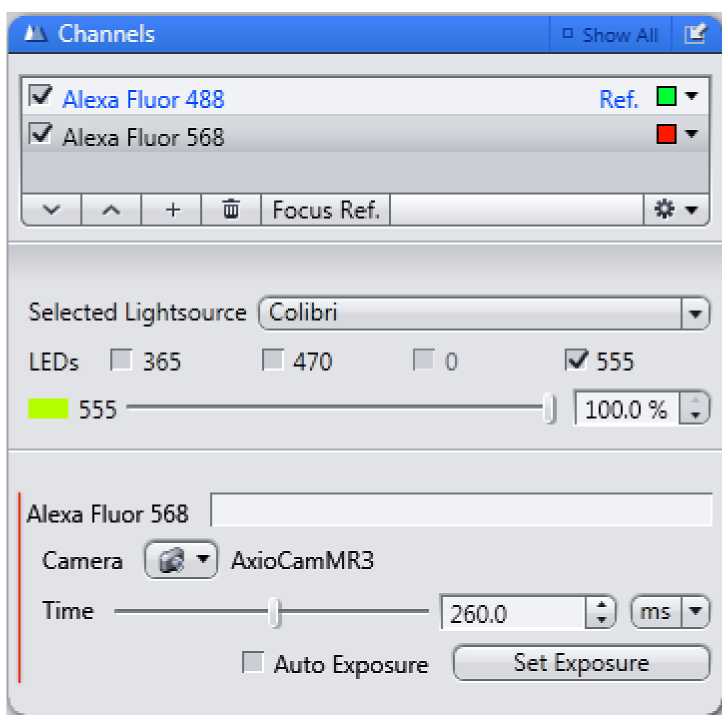
3. Open the **Smart Setup** .

4. Select the appropriate dyes (in our case Alexa 488 and Alexa 568):



5. Close the dialog by clicking on **OK**.

→ You will now see two channels in the **Channels** tool.

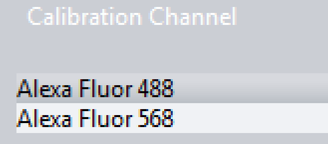
6. 

You have successfully set up the channels. In the live mode you can now check the focus and exposure time for the two channels.

14.2.7 Step 2: Grid focus calibration

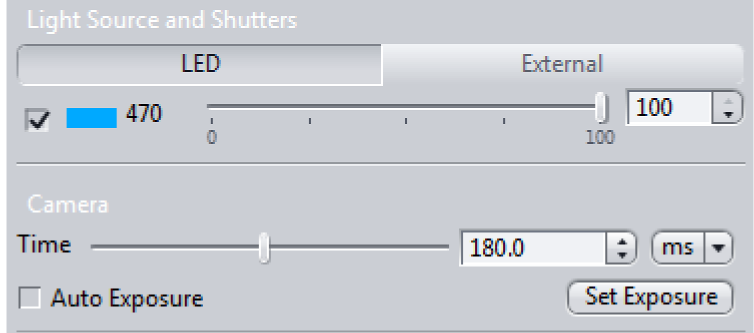
Aim

In this step you will calibrate the focus position of the ApoTome grid for the selected channels. This step is essential, as without a valid calibration it is not possible to perform an ApoTome experiment. Provided that no changes are made to the device settings that are important for calibration (objective, filter and illumination source, camera) the calibration remains valid for future experiments. We nevertheless recommend that you repeat the calibration from time to time, especially if the sample type you are analyzing changes. Calibration takes place in a wizard, which guides you through 3 simple steps.

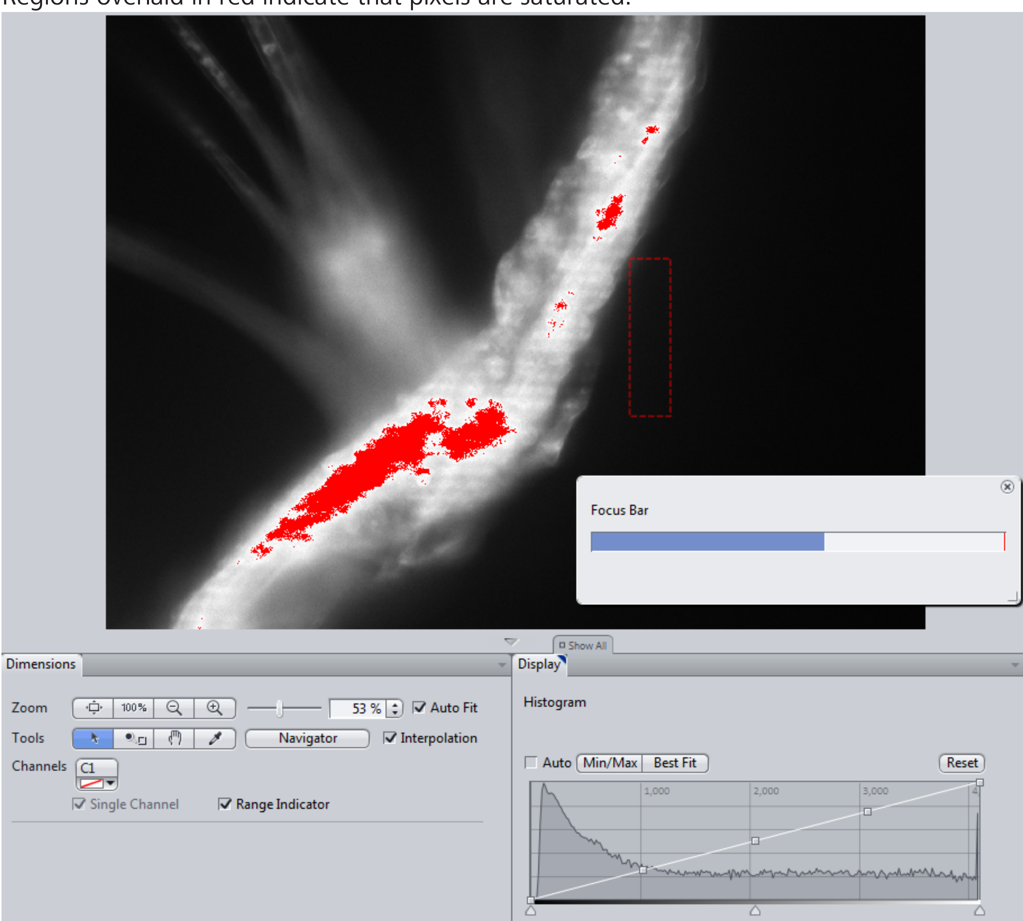
1. Move the ApoTome to the second click-stop position so the grid is positioned in the beam path.
2. From the **Acquisition** menu item open the **ApoTome Focus Calibration Wizard …** entry.
3. In the first step select the channel for which you want to perform calibration.


4. Click on **Next** to proceed.
   → You will now see the **Live Image**. Bring the sample into focus. The ApoTome grid has been moved to an end position to make it easier for you to focus on the sample. If you can nevertheless see the grid lines, something that can never be avoided entirely if you are using a 100x objective for example, click on one of the two buttons **Grid To Start** or **Grid To End** and use the position at which the fewest grid lines can be seen.

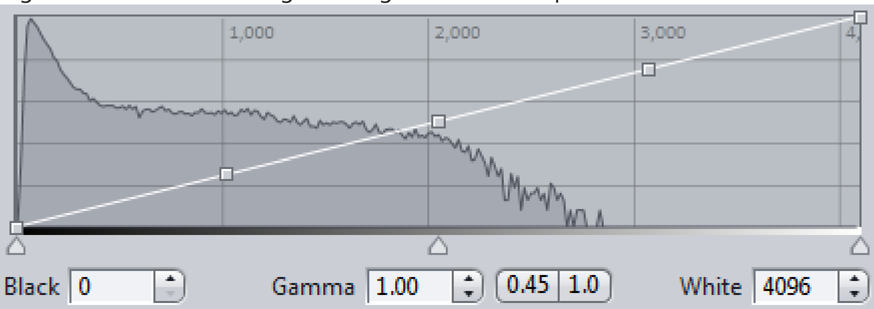
5. On the left you have the option of adjusting the exposure time and, if necessary, the illumination intensity (with corresponding light sources only).



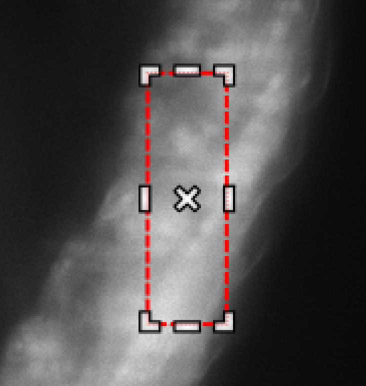
6. To adjust the exposure time correctly, select the saturation display on the **Dimensions** tab. Regions overlaid in red indicate that pixels are saturated.



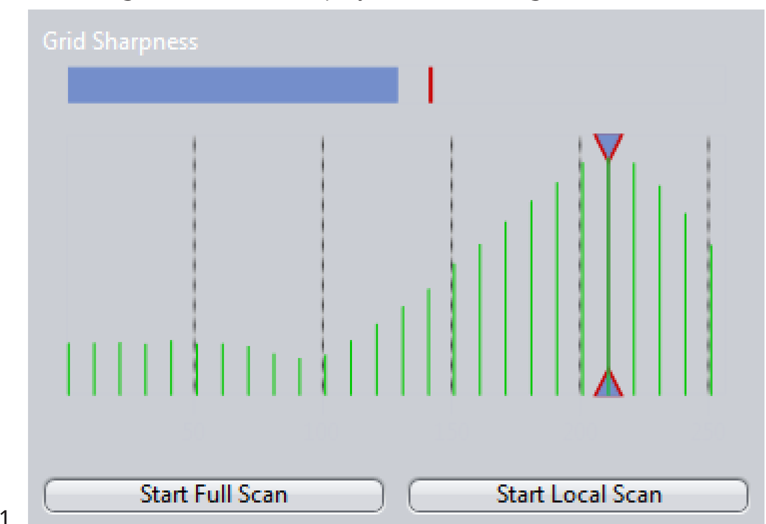
7. Reduce the exposure time accordingly. The ideal situation is where approx. 70% of the histogram is filled for the brightest region of the sample.



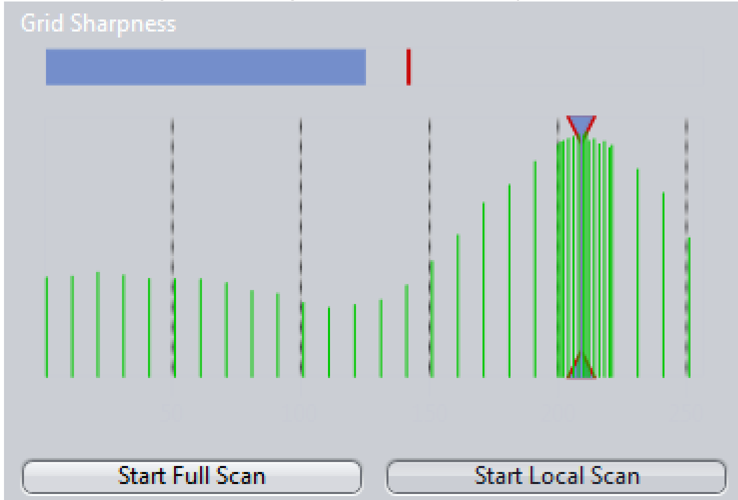
8. Position the rectangle in the live image and adjust its size in such a way that it covers fairly homogeneous fluorescent structures and does not lie over the background. The grid focus is only determined within this rectangle.



9. Click on **Next** to proceed to the final step.
10. Now click on the **Start Full Scan** button to start the grid focus search.
    → The grid contrast is displayed in the histogram.



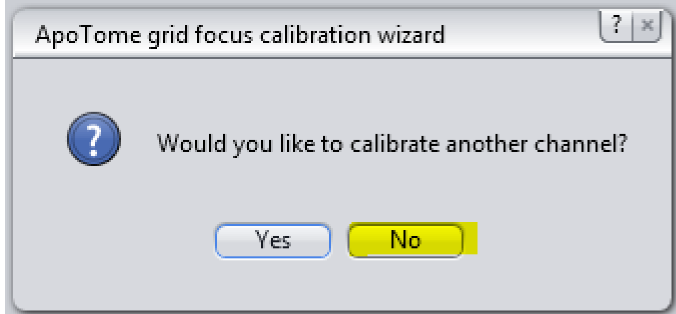
11. → More precise grid focusing can be achieved if you click on the **Start Local Scan** button.



However, this is only recommended for samples that are not particularly prone to bleaching. For samples prone to significant bleaching, the results of the **Local Scan** would measure considerably lower intensities and distort the result.

12. Click on the **Finish** button.

13. To perform calibration for another channel, answer **Yes** to the question "Do you want to calibrate another channel?" in the dialog that is now displayed.
    → The wizard then begins again from Step 1.
14. As soon as you have calibrated all channels, exit the wizard by clicking on **No**.



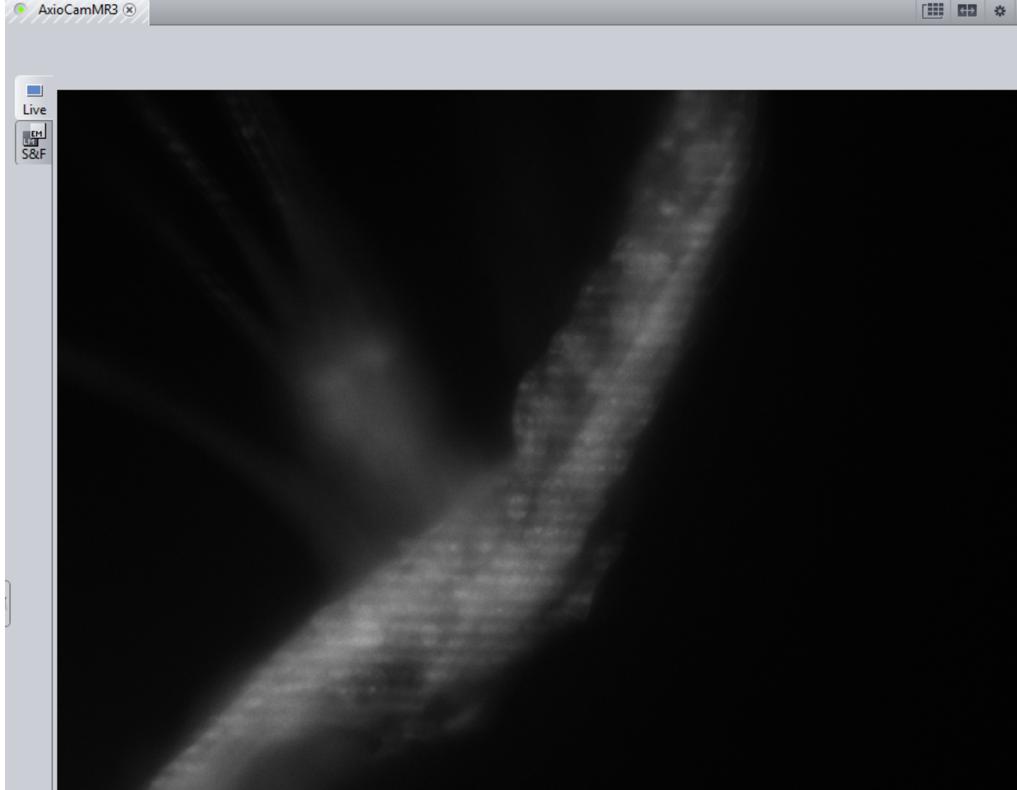
You have successfully performed grid focus calibration. Now continue with the next step.

14.2.8 Step 3: Perform ApoTome experiment

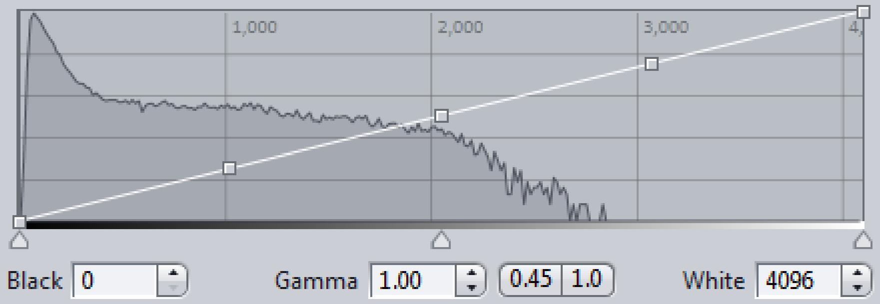
Aim

In this step you will perform acquisition for a two-channel experiment. You will use the same channels that were set up in Step 1. The objective must also be the same one used to calibrate the grid focus.

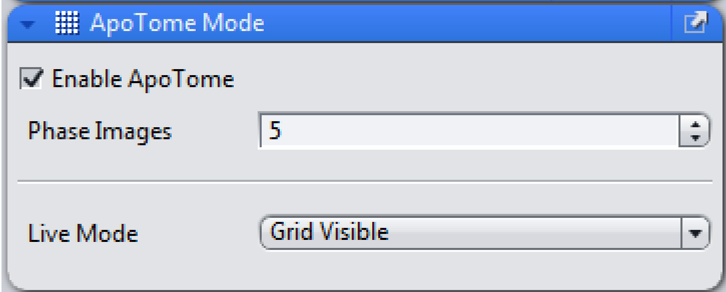
1. Insert the desired sample into the slide holder.
2. Open the **Live** Image and focus on the specimen.



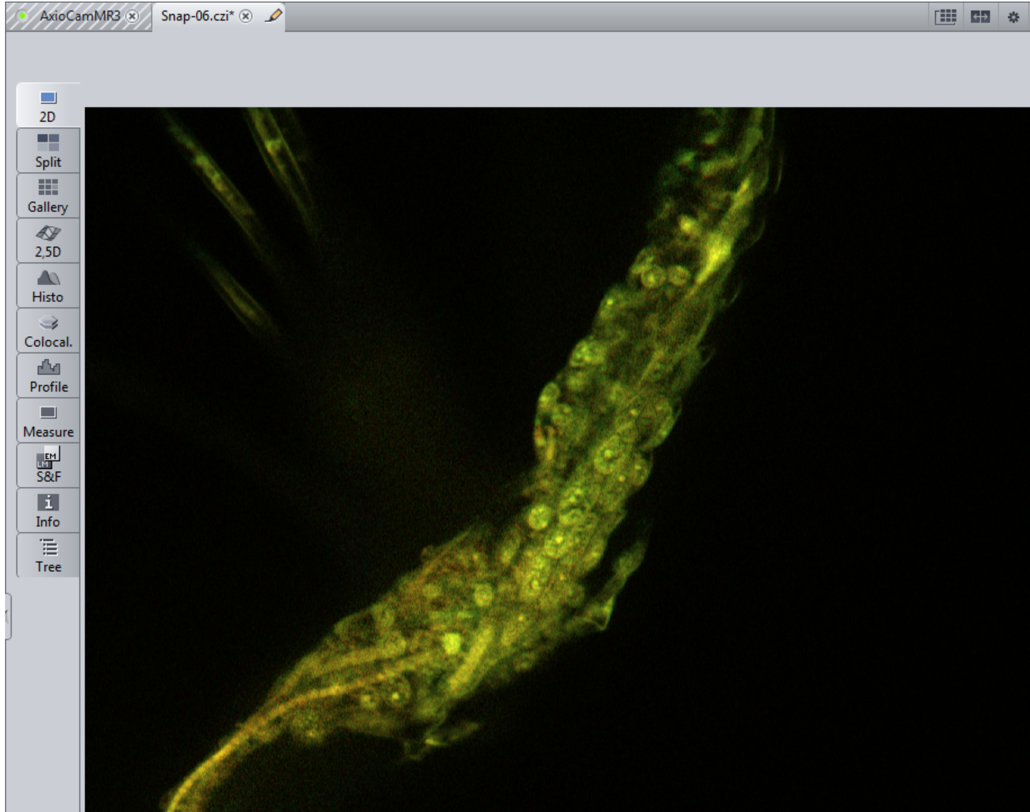
3. Adjust the exposure time for the two channels in such a way that approx. 70% of the **Histogram** is used.



4. Close the Live Image to protect the sample.
5. Open the **ApoTome Mode** tool on the **Acquisition** tab and enable the **ApoTome**.



6. Start acquisition by clicking on the **Snap** button.
   → The result is a two-channel image of your specimen.
7.



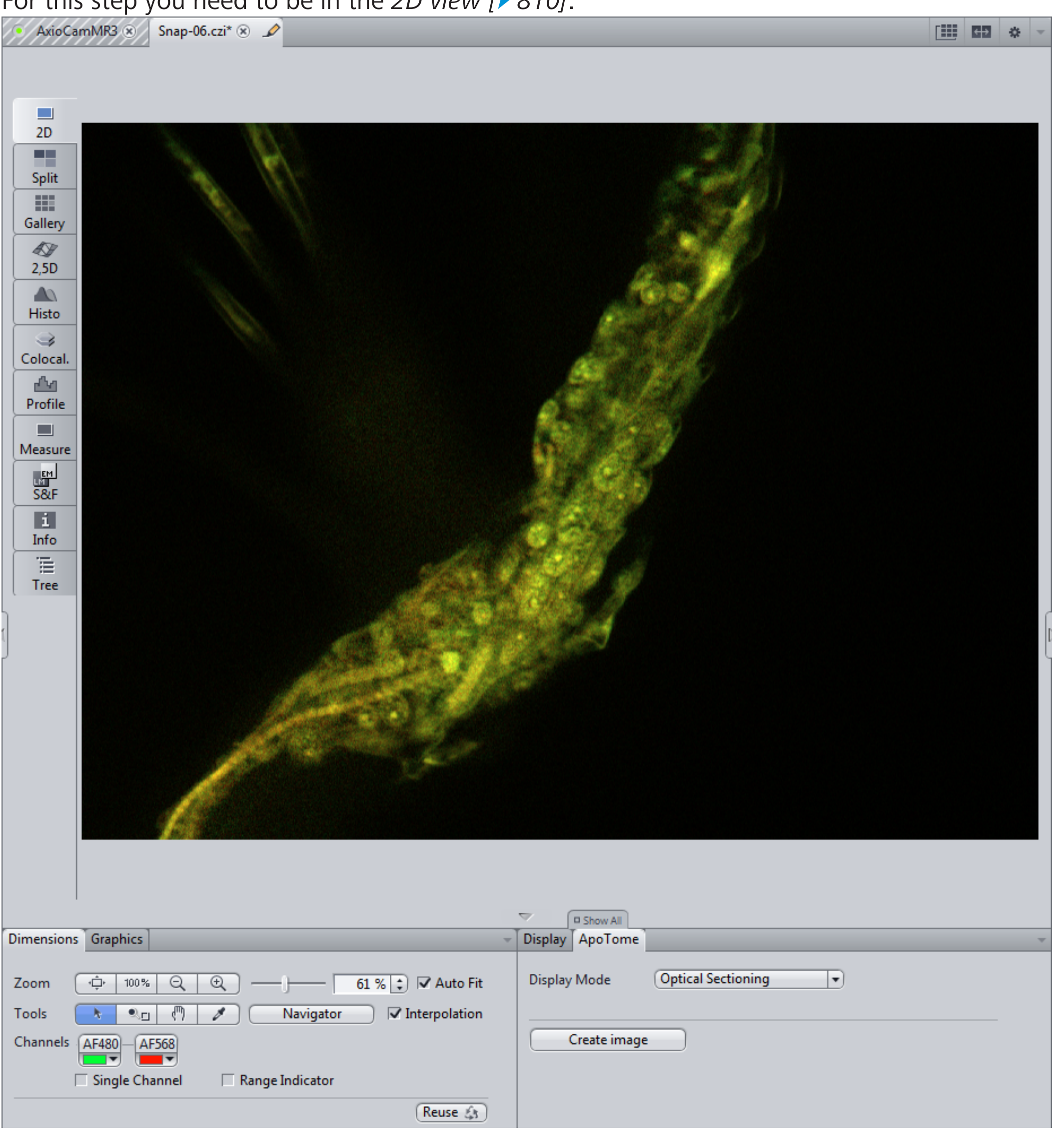
You have successfully performed an ApoTome experiment.

14.2.9 Step 4: Process the resulting image

Aim

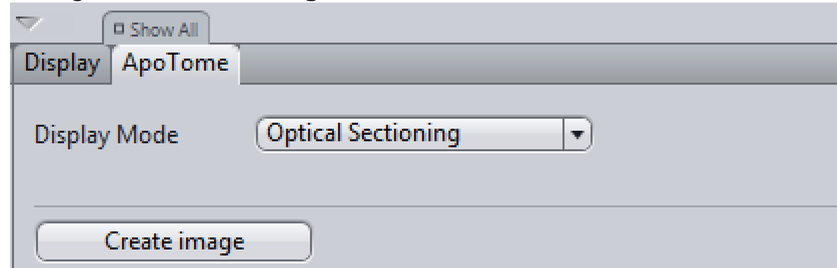
ApoTome images that are acquired from the **Acquisition** tab always take the form of raw data. In this step, with the help of the image you acquired in Step 3, we will look at the various display options available for ApoTome raw images. We will also create a processed resulting image, which you can process further as required.

Prerequisite ✓ For this step you need to be in the *2D view [▸ 810]*.



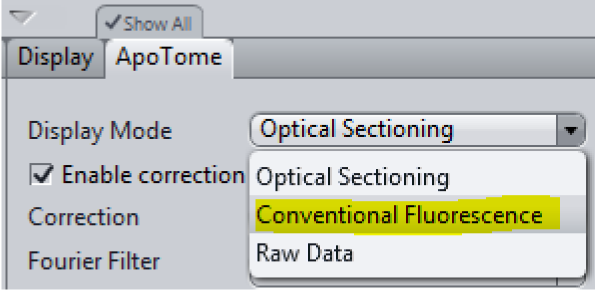
1. In the Center Screen Area go to the **ApoTome** tab. This view option is only displayed for ApoTome raw images.

→ If the Show all mode is deactivated you will see two view options: the **Display Mode** settings & the **Create Image** button.

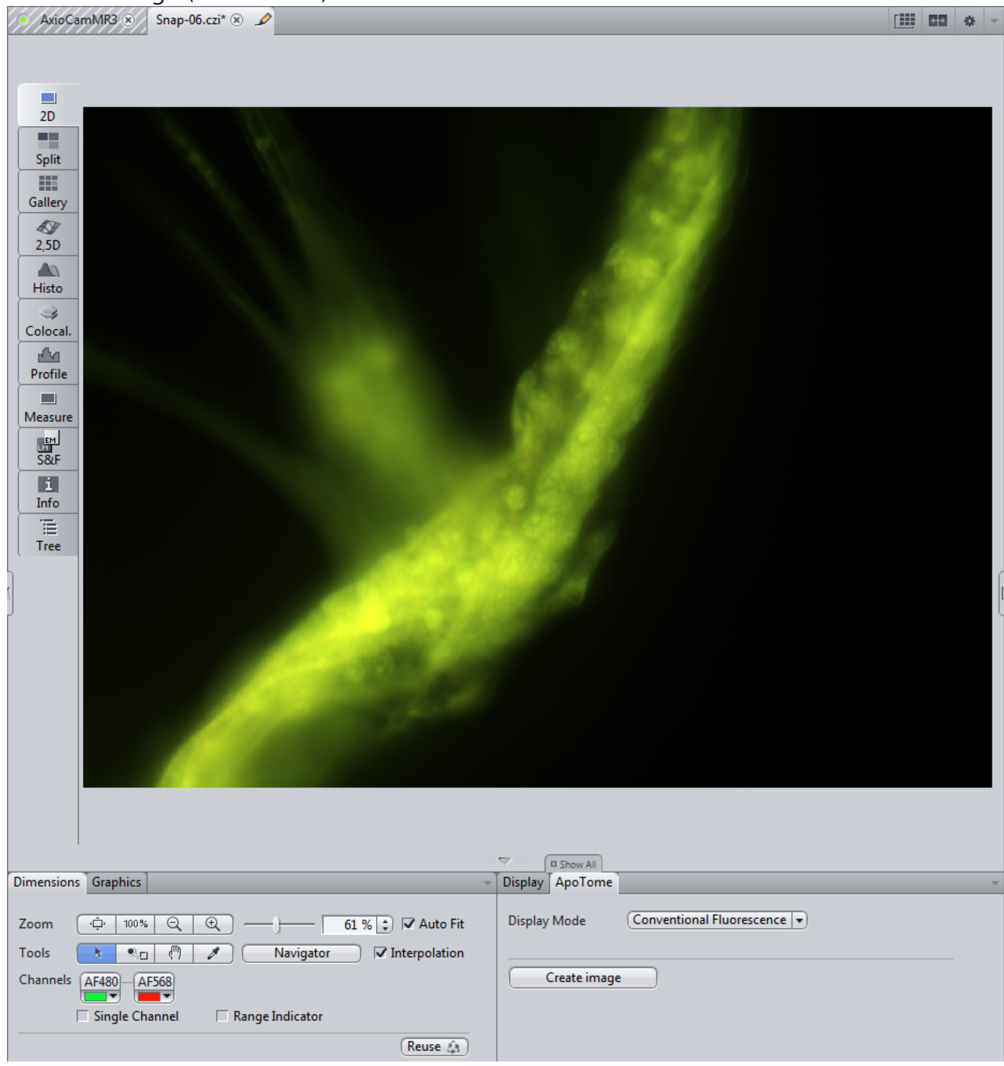


The default selection for Display Mode is the **Optical Section** view.

2. Select the **Conventional Fluorescence** option from the **Display Mode** dropdown list.



→ The image is now no longer displayed as an optical section, but as a conventional fluorescence image ("widefield"):



3. Activate the **Show All** mode to see additional settings for the calculation of the optical section image.

Show All
Display ApoTome
Display Mode Optical Sectioning Normalization
Enable correction
Correction Local bleaching Phase correction
Fourier Filter Off Grid 272.37 L/mm
Deconvolution
Create image

4. To see the difference between a corrected and uncorrected image, deactivate the **Enable Correction** option. Detailed information on the individual options can be found in the online help.
5. If you have not yet done so, enable the correction using the **Local Bleaching** option.
6. Create a new, processed resulting image by clicking on the **Create Image** button.

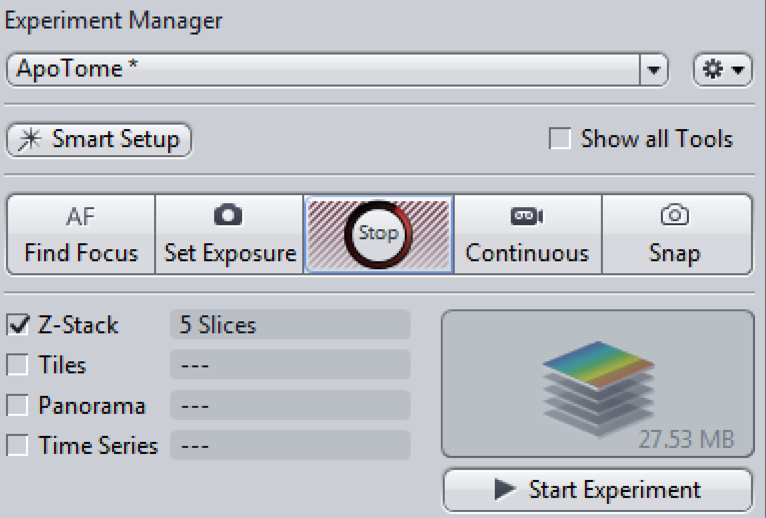
You have successfully processed the ApoTome image and created a resulting image for further processing.

14.2.10 Step 5: Perform Z-stack acquisition

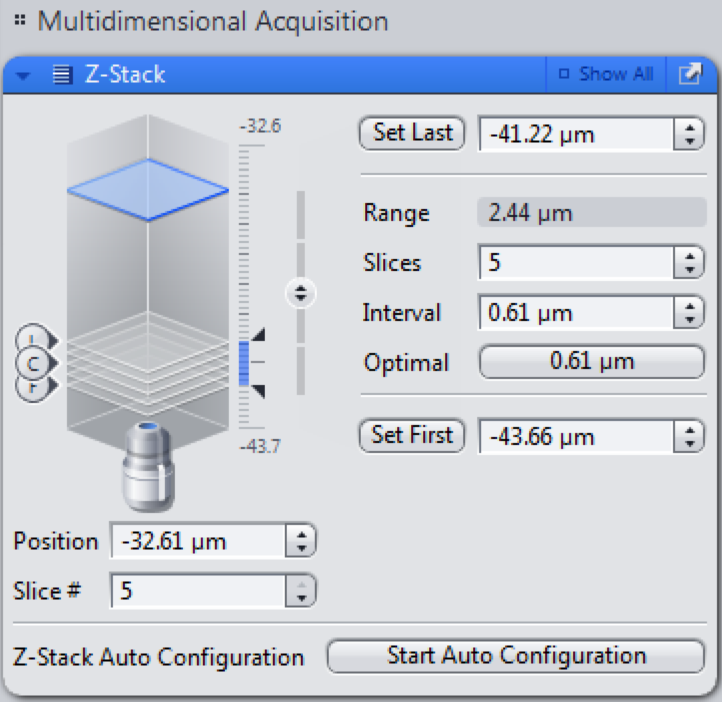
Aim

In this step you will acquire a Z-stack image with the same channel settings as in Step 3.

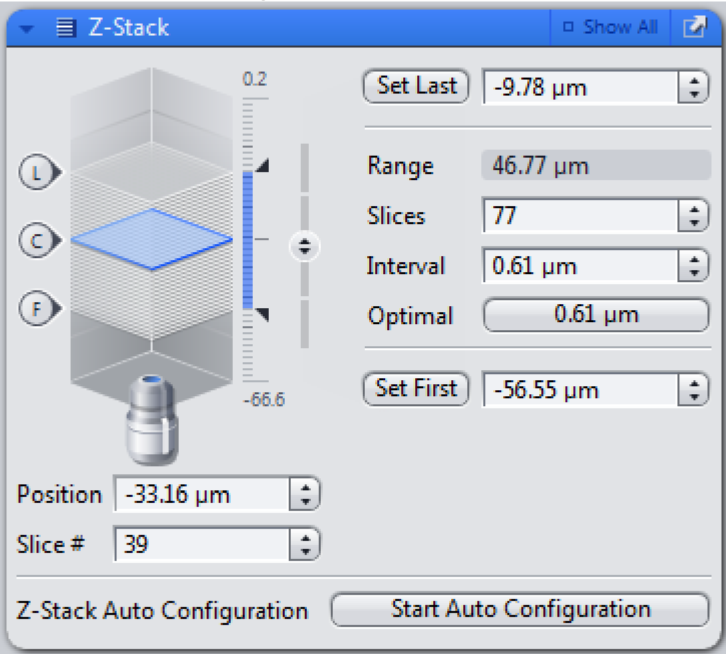
1. On the **Acquisition** tab activate the **Z-Stack** acquisition dimension in the Experiment Manager.



2. Open the Z-Stack tool.

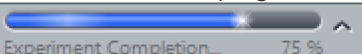


3. Start the **Live Mode** and define the dimensions of the Z-stack for your specimen. To capture the entire object three-dimensionally, you should set the upper and lower limit in such a way that object structures can no longer be seen in focus. Set the interval between the individual Z-planes using the **Optimal** button:



4. Start acquisition by clicking on the **Start Experiment** button.

→ You can follow the progress of the experiment in the progress bar on the status bar:



You have successfully acquired a Z-stack image. Save the resulting image under a meaningful name via the **File** menu | **Save** (**Ctrl+S**).

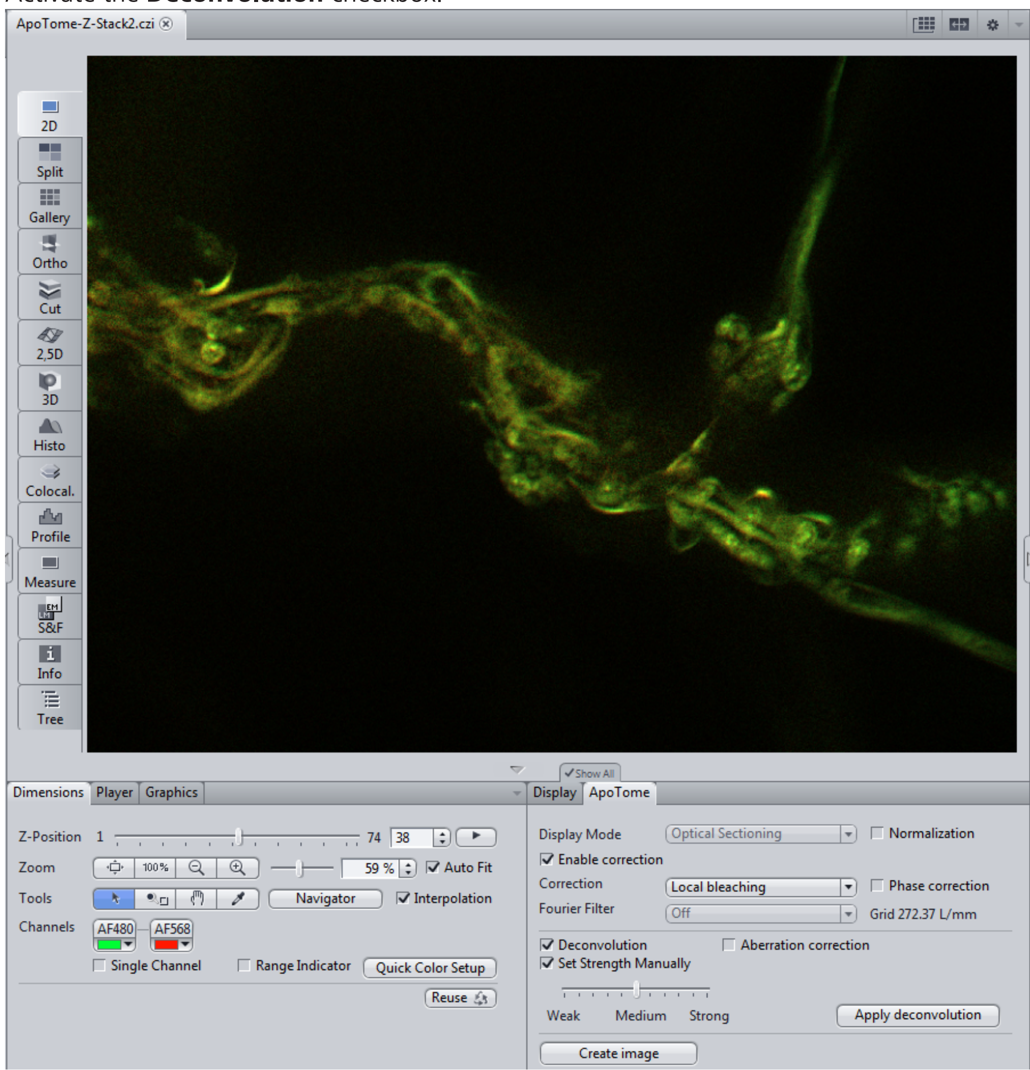
14.2.11 Step 6: Perform ApoTome deconvolution

Aim

In this step ApoTome deconvolution will be performed for the Z-stack acquired in Step 5. This enables you to significantly enhance the image, beyond what is possible using the normal **ApoTome** processing functions.

Prerequisite ✓ The Z-stack image must be in the foreground and in the 2D view. Go to the **ApoTome** tab (view option). Make sure that the **Show All** mode has been activated.

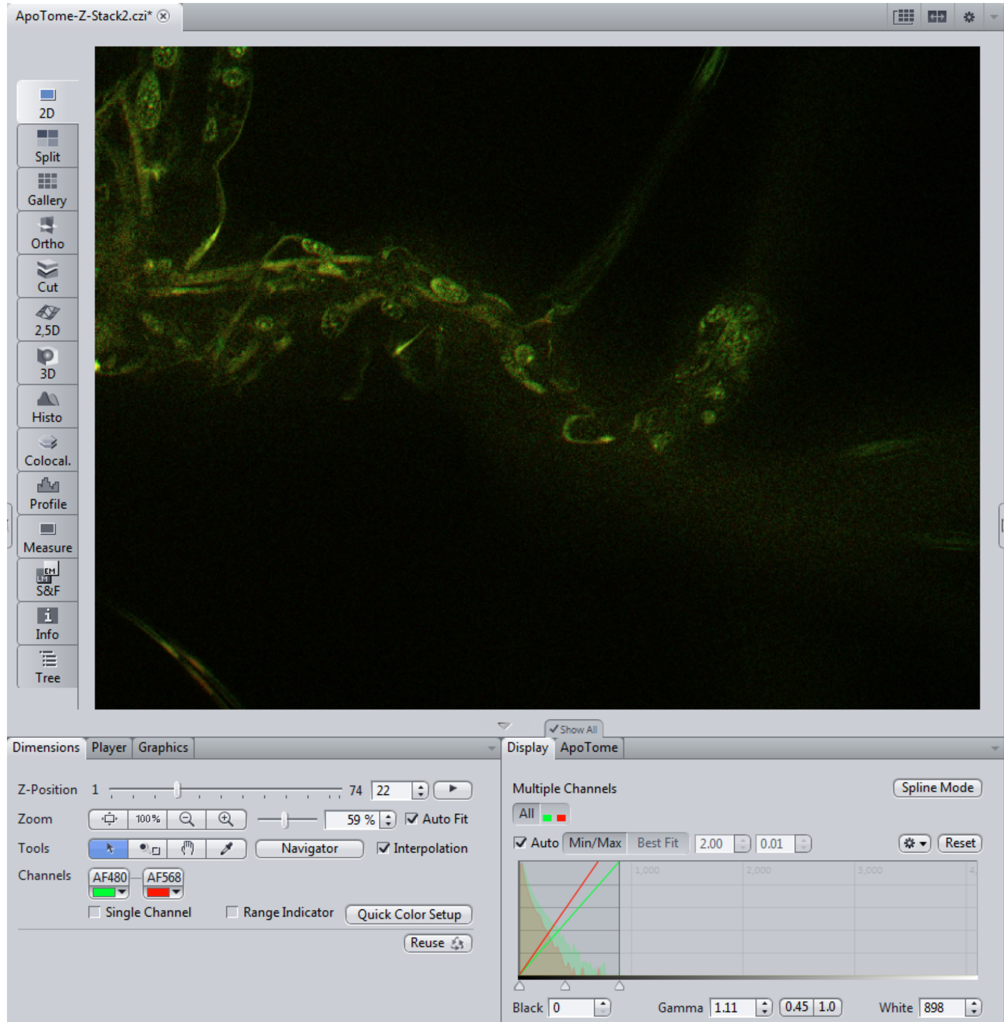
1. Activate the **Deconvolution** checkbox.



→ Make sure that the **Set Strength Manually** option is also activated. The **Strength** slider is set to **Medium** by default. Retain this setting for the time being.

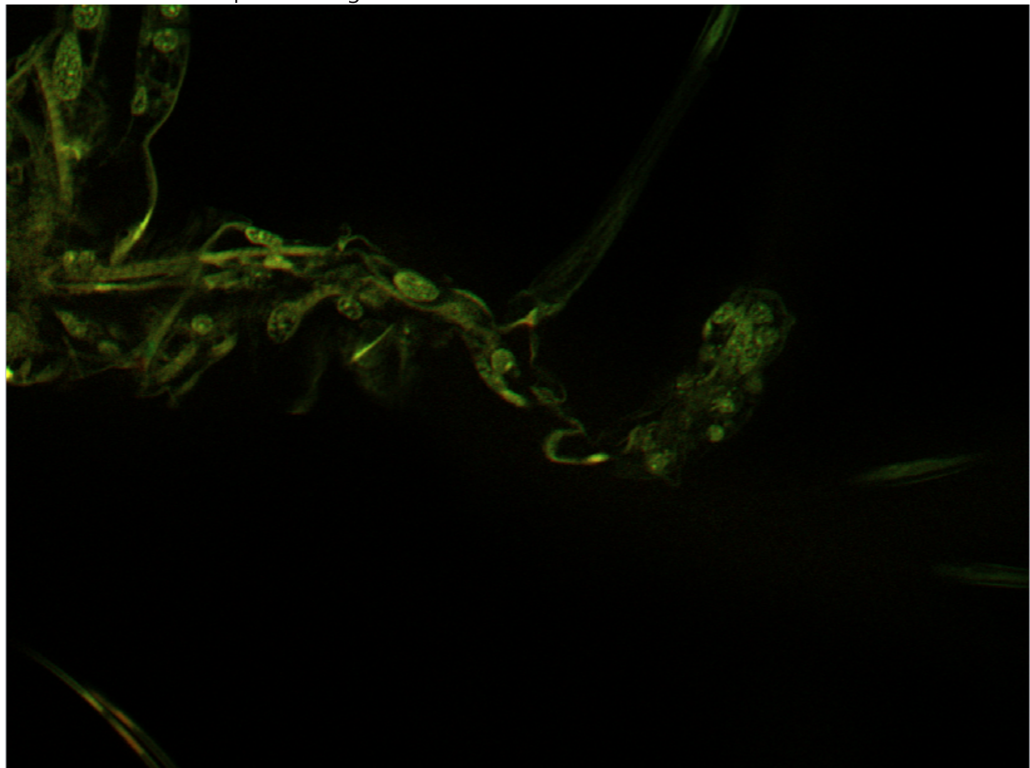
2. Click on the **Apply deconvolution** button.

→ Depending on the image size and the specifications of the computer, the processing can take anything between a few seconds and a few minutes. Make sure that you also adjust the brightness and contrast using the settings on the **Display** tab (tip: try out the **Min/ Max** button).

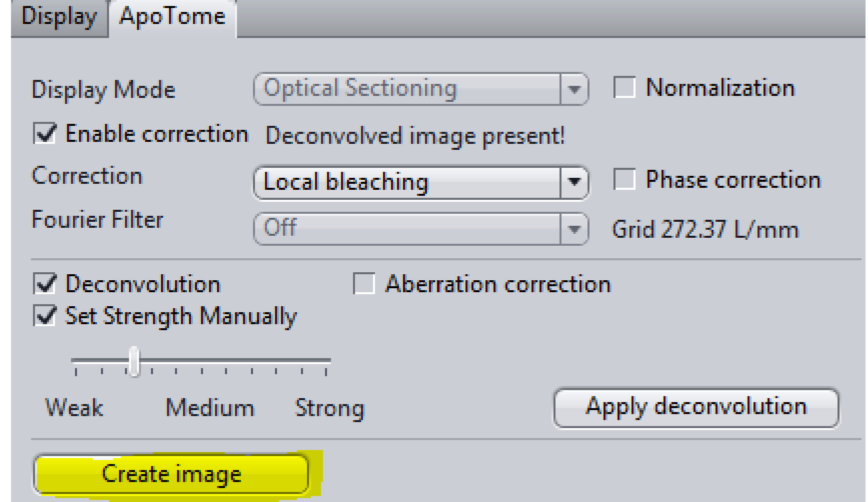


3. Examine the result by navigating through the Z-stack using the **Z-Position** slider on the **Dimensions** tab.

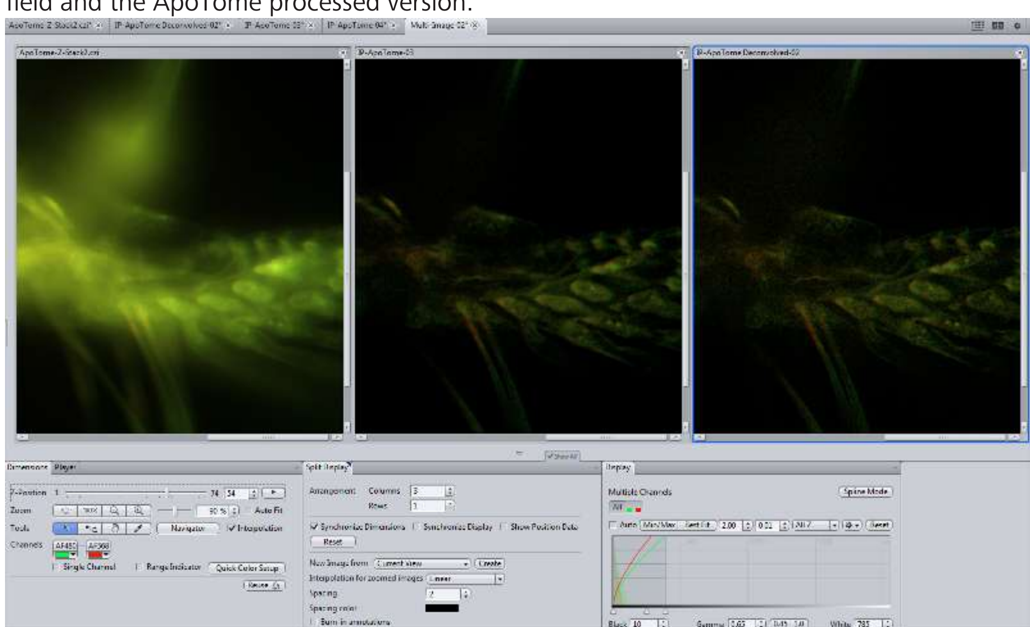
4. Change the **Strength** setting on the **ApoTome** tab until there is an obvious improvement and there is no disruptive background noise.



5. To obtain the result as a separate image document, click on the **Create image** button.



6. Using the *Splitter mode [909]* you can now compare the resulting image with the wide-field and the ApoTome processed version.



You have successfully performed ApoTome deconvolution, created a resulting image and compared different resulting images.

See also

ApoTome tab [905]

14.3 Celldiscoverer 7

14.3.1 Introduction

Celldiscoverer 7 - Your Automated Platform for Live Cell Imaging

The **Celldiscoverer 7** is an automated inverted imaging platform for research applications. The system calibrates itself, detects and focuses your samples and has adaptive optics to provide the optimal optical settings in an automated fashion. Thanks to its next generation opto-mechanical concept, a brand new line of dedicated objectives and a multitude of innovative built-in automation features **Celldiscoverer 7** delivers benchmark data quality and easy operation. It allows to work with a huge variety of sample carriers like wellplates, petri dishes and slides.

The system can automatically detect and calibrate the sample carrier category, measure the bottom material thickness and adapt the optics automatically to deliver maximum image quality. A built-in incubation chamber, auto immersion allow for long-term live cell imaging experiments. The new hardware based focus system can not only hold the focus over time but find the sample carrier surface.

The system is controlled by the ZEN Blue software package which offers all tools and functions you need to operate the **Celldiscoverer 7** efficiently and automate your imaging workflow. The software contains some additional functions allowing the user to navigate in a safe, easy and efficient way without unnecessary bleaching.

The following modules are part of the **ZEN Celldiscoverer** software:

- **Tiles & Positions** - in combination with focus strategies this allows for easy and flexible image acquisition especially for multi-position and multi wellplate experiments.
- **Advanced Processing & Image Analysis** - Use the built-in image analysis functions, create pipelines to run online image analysis and modify experiments based on those results on the fly.
- **Automation GUI** - Automate routine experiments and using scan profiles that can be started with just one button.
- **APEER (on-site) Basic** - On-site execution of APEER demo modules.
- **Experiment Designer** - Configuration of inhomogeneous acquisition experiments.
- **Extended Focus** - Calculation of a completely sharp 2D image out of a Z-stack.
- **Measurement** - Advanced interactive measurement tools.
- **Multi Channel** - Acquisition of fluorescence and transmitted light images in independent channels.
- **Software Autofocus** - Determination of the optimal focus position of the specimen.

- **Time Series** - Acquisition of time series.
- **Z-Stack** - Acquisition of Z-Stacks with the help of a motorized focus drive.

Additional ZEN modules are available as option:

- **Macro Environment** - Powerful Python scripts allowing to automate all kind of workflows, export data and connect to 3rd party application required for the workflow.
- **3Dxl** - Visualization of 3D or 4D image data.
- **Airyscan** - Processing of data acquired with Airyscan 2 (LSM only).
- **Airyscan 2 Basic** - Multiplex acquisition with 2x parallelisation (LSM 900 only).
- **APEER (on-site) Advanced** - On-site execution of individual APEER modules.
- **Automated Photomanipulation** - Acquisition of multiposition experiments with photomanipulation.
- **Colocalization** - Quantitative colocalization analysis between two fluorescence channels.
- **Connect** - Advanced functionality of ZEN Connect.
- **Connect 3D Add-on** - Extension for ZEN Connect for 3D workflows.
- **Data Storage Client** - Connection to ZEN Data Storage database.
- **Deconvolution** - Improvement of 3D image stacks via 3D-deconvolution algorithms.
- **Direct Processing** - Processing of images directly during acquisition.
- **FRAP** - Fluorescence Recovery after Photobleaching (FRAP) analysis.
- **Guided Acquisition** - Automatic and targeted acquisition of objects of interest.
- **HDR Confocal Basic** - High Dynamic Range (HDR) acquisition mode.
- **Intellesis** - Image segmentation based on machine-learning algorithm, using pixel classification.
- **Physiology (Dynamics)** - Analysis of physiological time series data.
- **Third Party Import** - Import of 3rd-party microscopy images into ZEN.

14.3.2 Sample Tab (Interactive mode)

The **Sample** tab is the central point of operating the **Celldiscoverer 7** system. On top of the tab you can activate two different modes, the **Interactive** or the **Automation** mode. Usually the Interactive mode is used for the default workflow when no plate loader is present or required. The Automation mode is especially suited for running routine experiments in an automated fashion. To learn more about the Automation mode, read *Sample Tab (Automation mode) [▸ 1017]*.

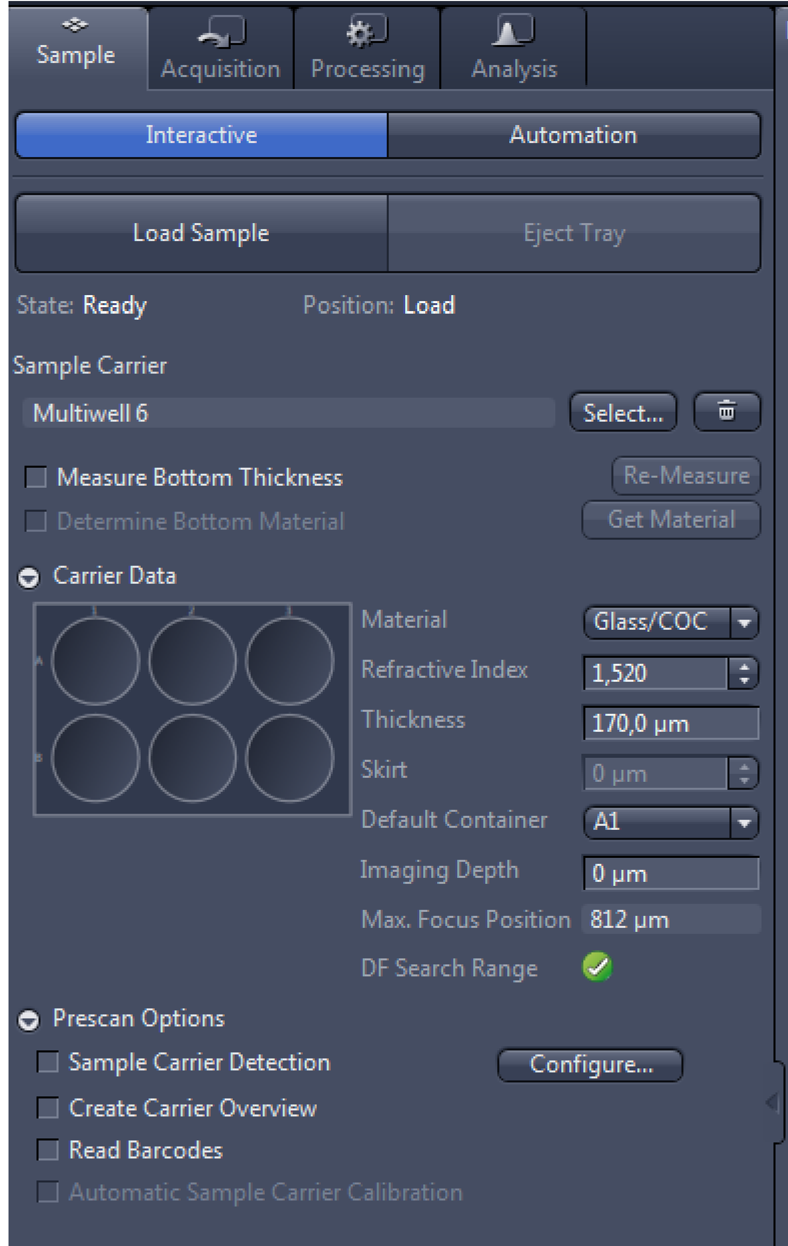


Fig. 115: Sample tab - Interactive mode

If you have selected **Interactive** mode, the following controls and functions are available:

Parameter	Description
Load Sample	Loads the inserted sample and performs various actions depending on the selected options under **Sample Carrier** and under **Prescan Options**. The detection of the bottom surface to measure the skirt of the sample carrier is always executed for every individual insert separately.

Parameter	Description
Eject Tray	Ejects the loaded tray with the sample carrier.
State	Shows the current system status.
Position	Shows the current position of the sample carrier. When the tray is ejected the position is Load, e.g. the system is ready to be loaded with a sample carrier. When the carrier is in scan position (above the objectives) the stated position depends on the current carrier type. As an example it could state **WellPlate** or **Petri Dish 6x, A1**.
Sample Carrier	
- Select...	Here you can select the sample carrier template you want to work with from list. Inside the selection dialog it is also possible to define and specify your own custom sample carriers, for example your favorite brand for a 384-well plate carrier. Be aware of the fact, that one must use the provided insert plates at any time in order to avoid collisions .Click on the **Delete** button to remove the selected sample carrier template. It is not possible to leave the selection empty, since the Celldiscoverer relies on having a valid sample carrier template all the time.
- Measure Bottom Thickness	**Activated**: The bottom thickness of the sample carrier is measured using special optics. Make sure the used sample carrier has the correct bottom material selected. When glass or COC is selected as a material the thickness measurement will have much smaller search range. In case of Polysterol as the selected material the search range will be large in any case. Note that the measurement may not be correct for embedded samples.
- Re-Measure	Performs the bottom thickness measurement again at the current XY position.
- Determine Bottom Material	**Activated**: Attempts to determine the use bottom material on demand. The method can differentiate Glass/Cycle Olefin Copolymer (COC) / "glass-like" materials and Polysterol (PS).
- Get Material	Performs the material determination again at the current XY position.
Carrier Data	After the sample carrier detection during the prescan (see **Prescan Options** below), a graphical sketch of the sample carrier data will be displayed here. Additionally detailed information about the carrier are shown. If the sample carrier detection option is not activated, you can enter the sample carrier data here manually e.g. **Material**, **Refractive Index**, **Thickness** etc..
- Material	Here you can select the correct bottom material, e.g. **Glass/COC** or **Polystyrene** (PS).
- Refractive Index	Here you can modify the pre-selected values if required.
- Thickness	Shows the measured bottom material thickness. You can adjust the value if required.

Parameter	Description
- Skirt	Per default this parameter can not be edited. You can switch it on under **Options** | **Celldiscoverer** | **General** | **Allow manual adaptation of skirt**. This value is measured automatically for every sample as soon as the tray is loaded. It defines the distance from the surface of the tray to the bottom surface of the actual carrier, e.g. the well plate.
- Default Container	Specifies the container which is approached initially, e.g. to detect cover glass thickness and measure the skirt height.
- Imaging Depth	Specifies the desired penetration depth for autocor objectives.
- Max. Focus Position	Shows the current upper z-limit of the focus drive.
- DF Search Range	This field shows whether the Definite Focus (DF) search range is restricted by the z-limit. If a warning is displayed you can do the following: ▪ Increase the z-limit (Under **Options** | **Celldiscoverer**) ▪ Reduce the imaging depth ▪ Check the refractive index
Prescan Options	
- Sample Carrier Detection	**Activated**: The system automatically detects which type sample carrier category is used. The result is displayed in the **Carrier Data** section. The **Configure** button allows to assign a special carrier template to carrier category. For example the Pre-Scan recognizes a 96 well plate (category). This "recognition event" can be assigned to sample carrier template "MyFavorite96Plate".
- Create Carrier Overview	**Activated**: An overview image of the sample carrier is acquired with the Pre-Scan camera. The overview image is displayed in the image document area after the Pre-Scan.
- Read Barcodes	**Activated**: The system automatically detects barcodes on the sample carrier. Currently the system can read codes placed on the short sidewalls of well plates or on top of the carrier, e.g. on a slide.
- Automatic Sample Carrier Calibration	**Activated**: An automatic sample carrier calibration is performed during pre-scan. This functionality is available for multiwell plates containing 48, 96, 384, or 1536 wells.

14.3.3 Using customized sample carriers

The Celldiscoverer 7 allows to create customized sample carrier templates, e.g. for IBIDI multi-chamber slides.

14.3.3.1 Designing a customized sample carrier template

This chapter describes an example of how to design a customized sample carrier template for multichamber slides.

1. Click on **Tools > Sample Carrier/Holder Templates**.
   → The **Templates** dialog opens.
2. Right click on **Slide 76 x 26 mm** and select **Copy and Edit**. It is important to select a slide. Otherwise the sample carrier detection will not work!
   → The dialog to edit the template opens.
3. Rename the template.
4. For **Category**, select **Multichamber**.
5. Add the correct dimensions of the sample carrier. It is possible to select the **Default Container** that is initially approached to automatically detect the sample carrier bottom material and thickness.
6. Click on **OK**.
7. Click on **x** to close the **Templates** dialog.

You have now created a customized sample carrier template.

See also

Combining the slide holder template with the custom template [▸ 1015]

14.3.3.2 Combining the slide holder template with the custom template

1. On the **Sample** tab, under **Sample Carrier**, click on **Select**.
2. In the **Select Template** dialog, right click on **Insert 2x Slide - Long** and select **Copy and Edit**.
3. Rename your sample carrier according to your designed template.
4. In the **Part of Insert Carrier** dropdown list, select your previously created template.
5. Click on **OK**.
6. In the **Select Template** dialog, select your template in the list and click on **OK**.

You have now combined a sample holder template with your custom template.

See also

Activating the Automatic Sample Carrier Detection for the customized sample carrier template [▸ 1016]

14.3.3.3 Activating the Automatic Sample Carrier Detection for the customized sample carrier template

1. On the **Sample** tab, under **Prescan Options**, activate **Sample Carrier Detection** and click on **Configure**.
2. In the **Configure Sample Carrier Detection** dialog, select **Insert 2x Slide –Long** and click on **Select**.
   → The **Select Template** dialog opens.
3. Under **Workgroup Templates**, select your customized sample carrier and click on **OK**.
4. Click on OK to exit the **Configure Sample Carrier Detection** dialog.

The **Sample Carrier Detection** will now associate the customized sample carrier template with the long slide holder. Until changed, every time the slide holder is detected, the customized sample carrier template will appear.

14.3.4 Sample Tab (Automation mode)

With the Automation mode it is possible to create scan profiles that combine all the pre-scan options with an actual ZEN experiment. The Automation mode can be used for special sample carrier inserts, e.g. for 2x Slide (long or short), 3x Slide or 6x dish.

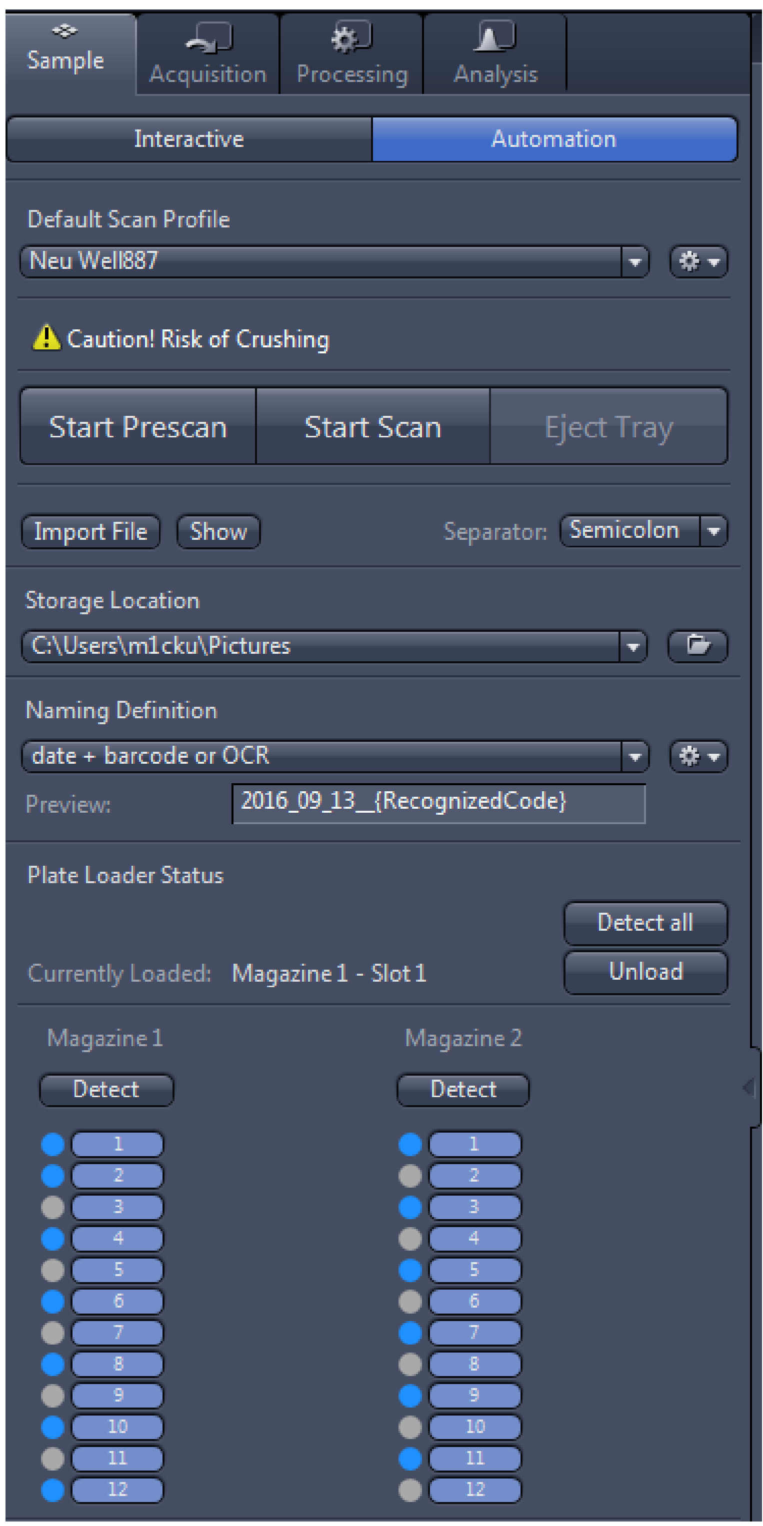


Fig. 116: Sample tab - Automation mode

If you have selected **Automation** mode, the following controls and functions are available:

Parameter	Description
Default Scan Profile	Here you can set a default scan profile. This profile will be used if new sample holders are inserted. If no profiles exists, you first have to create a new profile via the **Options** menu, see below:
- **Options**	▪ **New Scan Profile...** Creates a new scan profile. ▪ **Open Profile Configuration** Opens the *Profile Configuration [▸ 1020]* dialog. There you can edit an existing scan profile. ▪ **Rename** Renames the selected profile. ▪ **Save as** Saves the user profile under another file name. ▪ **Delete** Deletes the selected profile.
Start Prescan	Starts a prescan of all selected slots or the inserted sample (if no plate loader is used). To select a slot for a prescan, go to **Magazine** view and activate the corresponding checkbox in the **Process** column.
Start Scan	Starts the scan of all selected slots or the inserted sample.
Eject Tray	Ejects the tray which contains the sample holder.
Import File	If you click on this button you can import a file (*.csv format) which contains configuration data for the system. Typically the following data can be imported: ▪ **Magazine**, **Slot**, **Barcode**, **Scan Profile**, **ImageFileName**, **SubPath** Under **Separator** you can select the separator used in the *.csv file (e.g. Semicolon, Tab) If you click on '**Show**' a preview of the imported data will be displayed.
Storage Location	Here you can specify the storage location (file path) for all created images. All image sub paths, which might be read from the configuration file will be inserted below the defined file path.
Naming Definition	Here you can define naming definitions for the acquired images. You can select several definitions for the file names from the dropdown list. The name automatically contains the detected barcode content if the barcode detection is active for the active profile. Click on the **Options** button to define, edit or delete a definition.

Parameter	Description
Plate Loader Status	The display of this section depends on your system configuration. If you do not use a plate loader only the sample carrier which you have configured is displayed in the **Magazine** view. If you work with a plate loader, its magazine with the different slots is displayed. Common plate loaders can have up to 4 magazines. One magazine can contain up to 12 slots. In the slots you put your sample carriers containing the sample. The functions mentioned below only apply for the plate loader.
- Currently Loaded:	Shows the location (in the hotel of the loading robot) of the sample holder which is loaded currently.
- Detect All	Checks all magazines for slots which contain sample carriers automatically.
- Unload	Unloads the currently selected sample carrier from the Celldiscoverer back to its slot in the magazine.
- Reset	This button is only shown in case of an error with the plate loader (e.g. Plate loader is blocked). The respective message is shown in red. In this case all controls will be disabled and the plate loader will stop working. **NOTICE! In case of an physical obstruction, e.g. by a misplaced sample carrier, you first have to remove the sample carrier before you click on 'Reset'.** By clicking on **Reset** you can try to restore the system status of the plate loader. The system then tries to solve the problem using some internal error checking routines and additional actions.
Magazine 1 - ...	Here you see the graphical display of the magazine(s) and its lots. The magazines are numbered from 1 - 4 (depending on the available magazines). Under a magazine each slot is displayed as blue button. The slots are numbered as well (e.g. from 1- 12). The icon in front of a button shows the loading status. Following status are possible: ▪ gray = empty slot ▪ blue = occupied slot ▪ blue blinking = carrier is currently on the stage ▪ yellow = problem detected ▪ red = Error occurred during processing ▪ green blinking = Slot has status 'prescanned' ▪ green = Scanning the slot is finished. If you double-click on a slot button, the slot will be loaded or unload from the tray (depending of the loading status). If the tray is loaded with a carrier from a certain slot and you load a carrier from a different slot, the currently loaded slot will be unloaded first.

14.3.4.1 Profile Configuration

Parameter	Description
Selected Profile	Shows the name of the selected profile. Under **Profile Description** you can enter a short description, if desired.
Carrier Data	Contains information about the sample carrier
- **Automatic Carrier Detection**	In case of the option Automatic Carrier Detection the scan profile can contain data for different carrier types. They are visible inside the Sample Carrier list view.
- **Fix Carrier Assignment**	Only one specific carrier type is assigned to the scan profile. This is useful when working with the same carrier type all the time.
Options	
- **Create Carrier Overview**	Created and save an sample carrier overview image during the prescan which is store inside the actual image data file as an attachment.
- **Detect Occupied Positions**	In case of a sample carrier with multiple inserts, the systems automatically checks for empty positions.
- **Read Barcodes**	Read the barcodes from the respective position on the sample carrier itself (position depends on the carrier).
- **Use configuration file**	Three different options available: ▪ **No configuration file**: no configuration will be used. ▪ **Imported Data** : use a configuration that must be imported before. ▪ **Control Barcode**: Defines rules how a barcode will be interpreted.
Sample Carriers	This list view allows managing the sample carrier types supported by the current scan profile. The **+** button allows to add more carrier types. The **Delete** button deletes the carrier type including all assigned scan experiments.
Carrier Configuration	This area shows the respective carrier configurations and allows to modify them. For normal wellplates there will be only one data set visible, but for carriers with multiple inserts it is possible to define different configuration for every available insert
- Use same sample carrier configuration for all positions	When activated the same configuration will be used for all sample carriers during the processing.
Assigned Scan Experiments	Here you can assign the actual ZEN experiments. For carriers with multiple inserts it is possible to assign an individual experiment to every insert, e.g. it is possible to run a different experiment for every petri dish when using the 6x petri dish.
- Use same scan experiment for all positions	Allows assigning the same experiment to every individual position of the carrier, e.g. use the same experiment for all petri dishes of a 6x petri dish holder.

14.3.5 Magazine View

In general the **Magazine** view is used to get an overview of the status of the sample carrier(s) / scan items in the tray of the system or in the magazine(s) of the plate loader.

The display of this view depends strongly on the system configuration. If you use the system without a plate loader the configured sample carrier is displayed.

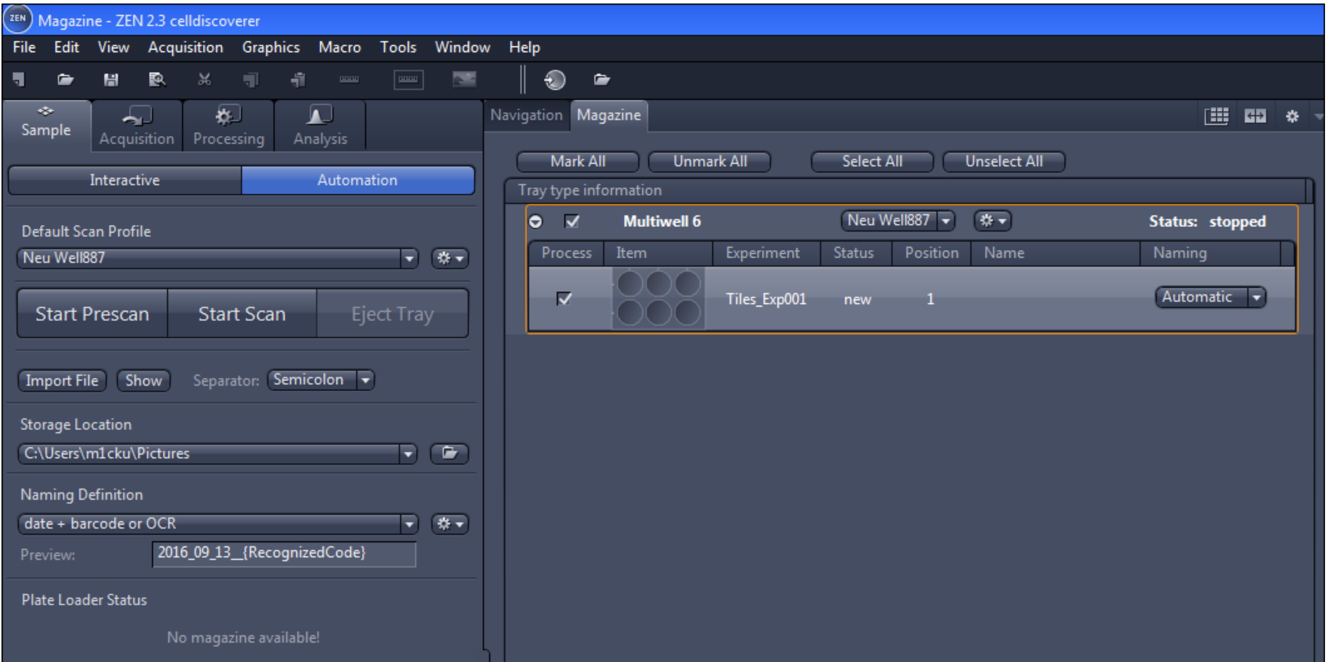


Fig. 117: Magazine View without Plate Loader

If you work with a plate loader you will see a list of the available magazines and slots. Note that only slots which are occupied are displayed. Empty slots are not displayed in the list. We recommend to perform a check for occupied slots by clicking on **Detect all** in **Sample** tab under **Plate Loader Status**.

The currently loaded slot / scan item is marked by an orange rectangle.

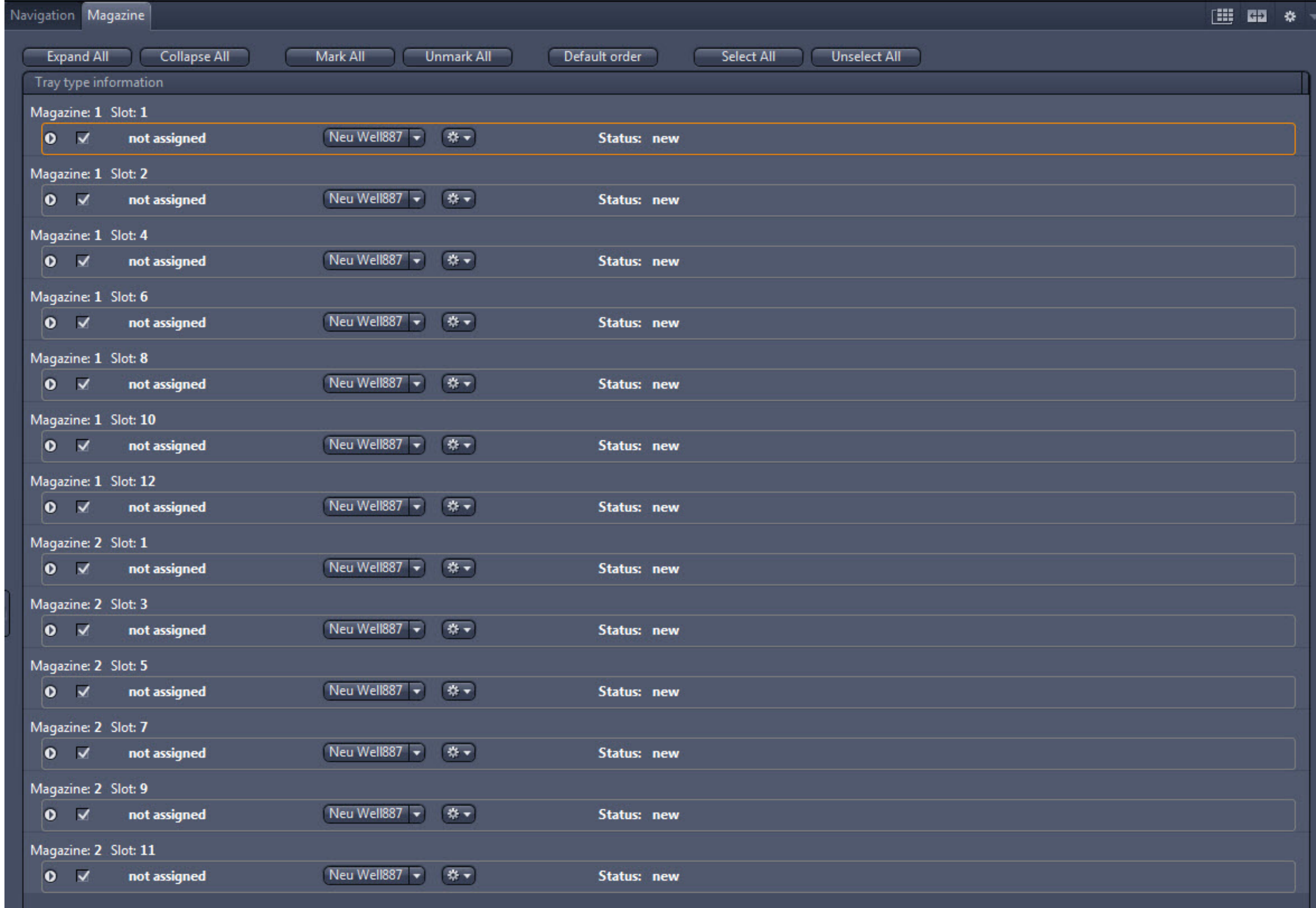


Fig. 118: Magazine View with Plate Loader

The following controls are available for Magazine view:

Parameter	Description
Expand All	Expands all scan items so that all of them are visible including there configurations. (This button is only visible when a plate loader is present.)
Collapse All	Collapses all scan items so that only the short information overview and the processing status is visible. (This button is only visible when a plate loader is present.)
Mark All	Activates the processing markers for all scan items. When clicking '**Start Prescan**' or '**Start Scan**' those items will be processed, if their status allows for those actions (new, prescanned, stopped).
Unmark All	Deactivates all processing markers.
Default Order	Restores the default order of slots. Their order can be changes via Drag & Drop. Newly detected carriers will be added at the end.
Select All	Selects all items and allows the execution of a context menu entry for all shown elements at once.
Unselect All	Unselects all items.
Tray type informa-tion	

Parameter	Description
- Show Information	Shows the results of the processing, if existing.
- Show Profile	Open a XML viewer to inspect the complete scan profile configuration.

14.3.5.1 Magazine View Options

The options below are opened via right click on the corresponding slot (e.g. Magazine:1 Slot:1).

Parameter	Description
Assign Scan Profile	Open a list with all available scan profiles. A click on the desired profile assigns it to the currently selected items.
Mark all scan items of highlighted trays for processing	Activates the processing checkbox for all selected slots and their scan items.
Unmark all scan items of highlighted trays for processing	Deactivates the processing checkbox for all selected slots and their scan items.
Show Overview Image	Opens the sample carrier overview image.
Expand all highlighted holders	Opens the sample carrier overview image.
Collapse all highlighted holders	Opens all selected sample carriers.
Reset carrier status to 'New'	Closes all selected sample carriers.

The options below are opened via right click on an individual sample carrier inside the list (scan item).

Parameter	Description
Move to Scan Position	Moves the stage to the respective scan position. Depends from the sample carrier and a possible insert and therefor from the scan item type.
Mark all highlighted scan items for processing	Opens the resulting scan image as an individual document inside the normal document area.
Unmark all highlighted scan items for processing	Resets all precessing checkboxes for all selected scan items.
Reset Scan status to New	Resets the status of the scan items to "new".

14.3.6 Navigation View

In the **Navigation** view you see the graphical display of the loaded sample carrier. In general the view is used for navigation purposes. Depending on the carrier, on **Selection** tab you can navigate to the single containers of the carrier (e.g. of a petri dish) simply by double clicking on the desired container. The stage will move automatically to the center of the selected container. In case of carriers with multiple inserts (e.g. 6x petri dish insert) the selection tab allows you the switch between the different inserts.

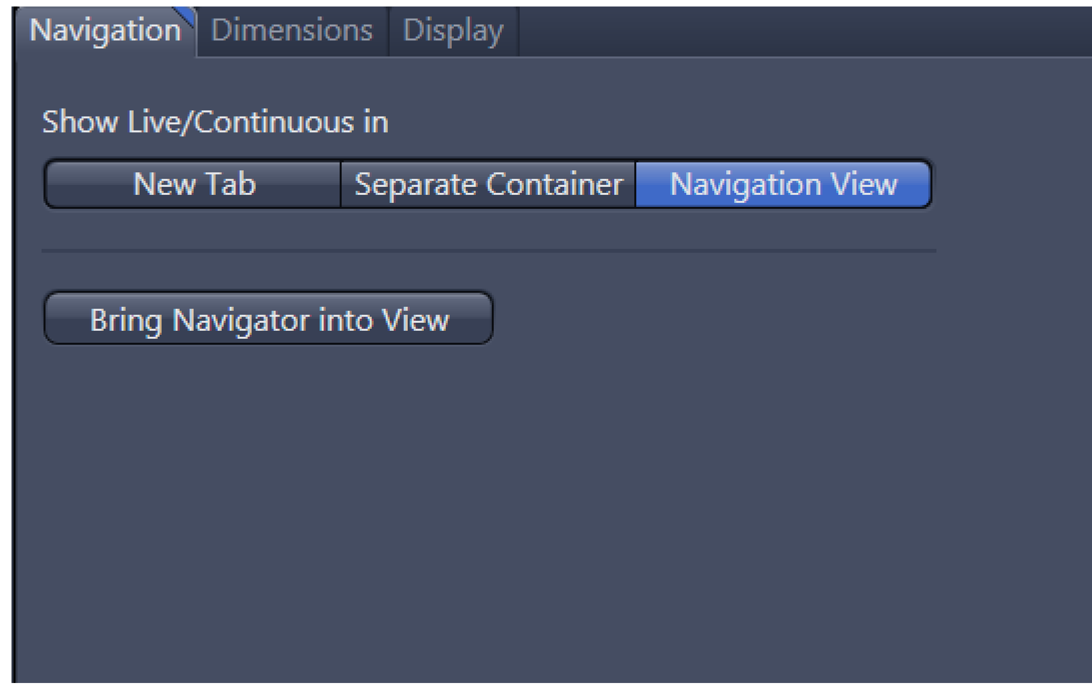
Fig. 119: Navigation view

The system is equipped with an automatic collision prevention system. Based on the geometry of the objective front, the working distance of the objective, the actual carrier or insert geometry and the skirt height the safe XYZ traveling ranges will be calculated and adapted automatically. In **Navigation** view the currently possible XY travelling ranges are always displayed. The current active limit for the selected objective is displayed by a thick, dashed line using the respective color. The limits for the other objectives are also shown using a thin line using the respective color.

In case that the travel range should be increased (especially for the high magnification objectives), the **Z-Limit above Surface** (**Tools > Options > Celldiscoverer**) can be decreased. See also the chapter *Celldiscoverer Options [▸ 1029]*.

The blue cross hair shows the current stage position and the blue rectangle (only visible at higher zoom levels) shows the field of view of the objective.

14.3.7 Navigation tab



Parameter	Description
New Tab	Live/continuous image will be displayed in a new tab.
Separate Container	Live/continuous image will be displayed in a separate container.
Navigation View	Live/continuous image will be displayed in the Navigation window (very small).
Bring Navigator into View	Enlarges the live/continuous image to full view inside the Navigation window (works for all three options **New Tab**, **Separate Container**, **Navigation View**).

14.3.8 Celldiscoverer Tool

The **Celldiscoverer** tool is located in the **Right Tool Area**. Note that the tool is not visible in **Automation** mode.

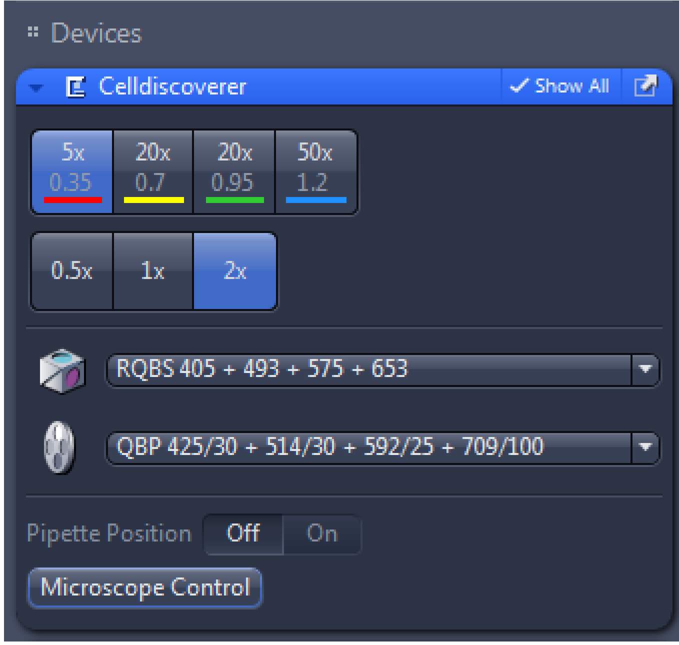
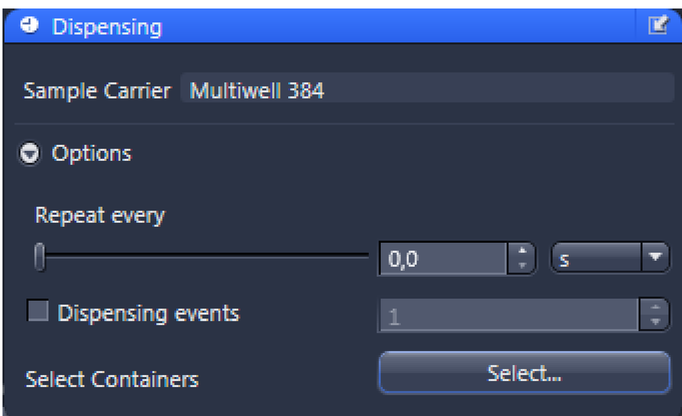


Fig. 120: Celldiscoverer tool

The tool is used for controlling the systems hardware components, like objectives, beam splitter, filter wheels, light path etc..

Parameter	Description
Active Camera	Here you can select the active camera or detector. The respective port switcher moves automatically.
Objective List	Here you can easily switch between the objectives and pre-magnification. The color bar on the objective buttons indicates the color for the respective stage limit indicator inside the **Navigation** tab. If you select AutoCorr objectives (motorized correction collar) you can additionally adjust the relevant settings like **Correction Mode, Bottom Thickness** or **Imaging Depth**.
Beam Splitter List	Here you can select the desired beam splitter from the list. If you change the beam splitter the corresponding emission filter from the list below is changed as well.
Filter List	Here you can select the desired emission filter from the list. A change here will not affect the selected beam splitter.
Pipette Position	If you click on the ON button the system moves in the pipette position, where it is possible to add reagents to the sample. The tip of the pipette will be located at the center of the optical axis indicated by the blue crosshair inside the **Navigation** tab. Make sure the height adjustment for the pipette tool is correctly adjusted to the current carrier geometry.
Microscope Control	Opens the **Microscope Control** dialog. There you can see and adjust the full light path of the system. We recommend to only adjust settings in the light path, if you know what you are doing.

14.3.9 Dispensing Tool



This tool is used to set up dispensing events for certain container(s) on a sample carrier. To perform dispensing, the system moves the sample carrier to the dispensing position where you can easily add dispensing fluid to the selected container(s). The **Dispensing** checkbox is displayed by activating **Time Series**.

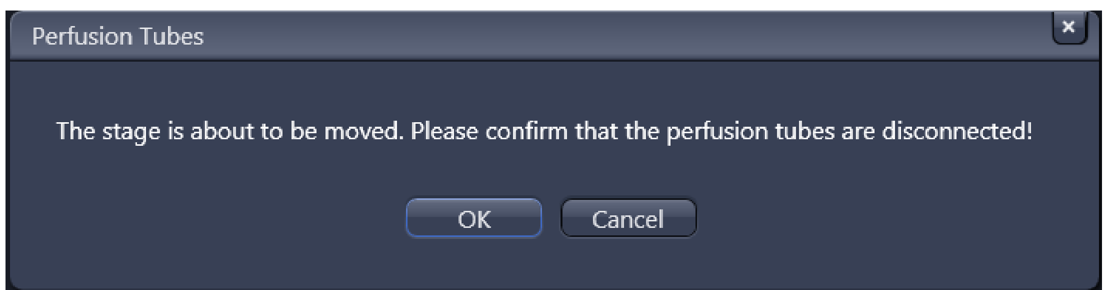
Parameter		Description
Sample Carrier		Shows the selected sample carrier which will be used for dispensing.
Options		
-	Repeat every	Here you can adjust the interval between dispensing events during an experiment. E.g. if you adjust the value to 5 min, the software will pause the running experiment after 5 minutes and allows you to add a substance to your sample.
-	Dispensing events	Here you can adjust the total number of dispensing events for an experiment.
-	Select containers	If you click on the **Select** button, the **Container selection** dialog opens. There you can select the containers in which you want to add the fluid.

Fig. 121: Container Selection Dialog

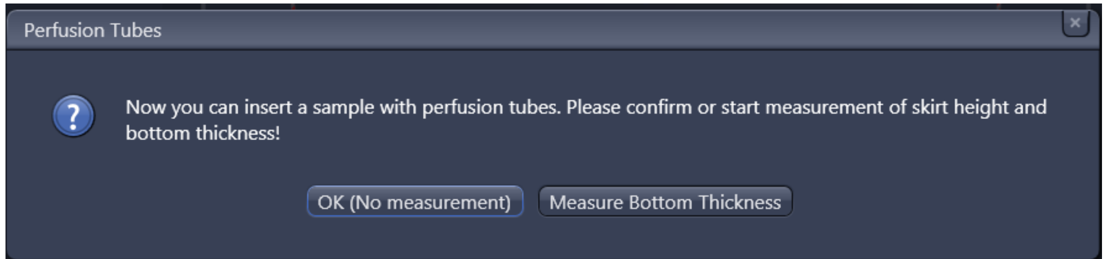
14.3.10 Perfusion

To enable perfusion experiments, insert the POC-R dispensing chamber into the Insert plate for perfusion.

Do not connect the perfusion tubes when loading the sample to avoid any damage.



If the sample has reached the imaging position, the perfusion tubes can be connected. By clicking **Measure Bottom Thickness** the skirt height and bottom thickness are determined.



14.3.11 Celldiscoverer Options

The Celldiscoverer **Options** menu can be found under **Tools** | **Options** | **Celldiscoverer**.

Parameter	Description
General	
- Use Automated Camera Frame Correction	When using the 0.5X after-magnification the chip window on the camera is adjusted automatically in order to remove the dark areas inside the corners due to the limited FOV for (only!) this after-magnification.
- Enable Automation Mode (restart required)	If activated and you work with a plate loader, on **Sample** tab the **Automation** mode is automatically activated.
- Disable empty check for automation	**Activated** In **Automation Mode** the sample is loaded without **Automatic Sample Carrier Detection**. We recommend this option if there are problems with the **Automatic Sample Carrier Detection** in **Automation Mode**. The sample carrier type has to be defined in advance.
- Allow manual skirt adaption	**Activated** (not recommended): the skirt height of the carrier can be adapted manually. Caution: This is not recommended! Please modify the skirt height only if it is known exactly. Otherwise, there is the risk of crushing the objective into the sample carrier.
- Ask User to Adapt Experiment with Inserted Sample Carrier	**Activated:** Shows a warning during loading of the sample carrier when the sample carrier does not fit to the actual experiment. Allows either to adapt the sample carrier or to leave the experiment unchanged. **Caution:** The adaptation of the experiment to the actual sample carrier will delete all tile regions/positions.
- Don't show a 'Warning' at experiment execution, if the validation result can be ignored	**Activated**: Shows no warning during experiment execution. Activating this is recommended for automation/execution of macros etc. to avoid stopping the experiment/workflow. ZEN sometimes asks for manual confirmation and this would cause the experiment or the macro to stop.

Parameter	Description
Cover Glass Thickness Detection Options	
- Use Default Thickness Detection (recommended)	If deactivated (**not recommended**), you can adjust the cover glass thickness detection options manually for trouble shooting.
- Use Aberration Correction	**Activated**: Attempts to improve the result of the thickness measurement by considering aberration effects. Per default this setting is deactivated.
Prescan Options	
- Show Images Used for Recognition	**Activated**: Displays the images used for recognition inside the Document area after the Pre-Scan. This option should be only activated for trouble-shooting the Pre-Scan sample carrier recognition. Normally this option can be deactivated.
- Show live container during sample carrier calibration	**Activated**: Displays the live images used for the automatic sample carrier calibration inside the Document area after the Pre-Scan. This option should be only activated for trouble-shooting the Pre-Scan sample carriers recognition. Normally this option can be deactivated.
Z-Limit above Surface	Allows adjusting the default Z-Limits for different objectives to increase the available XY traveling range. The icons inside the DF (Definite Focus) Search Range column indicate if the available Z-range is sufficient for performing a **Find Surface** operation.
Auto Immersion Refill Options	
- Remove Water	If you want to use Auto Immersion for an experiment, here you adjust the duration for the removal of the applied water. The set time defines the duration for which the suction pump is switched on (default: 5000 ms). For use of Auto Immersion, in the **Right Tool Area > Auto Immersion**, activate **Enable Immersion**.
- Apply Water	If you want to use Auto Immersion for an experiment, here you adjust the duration for the application of water (default: 2500 ms).
- Waiting Time after Refill	Sets the waiting time after a refill (default: 2000 ms).
- Refill every	Regardless of an experiment, here you adjust the interval time of the global periodic timer for renewing the immersion (default: 45 minutes). This prevents the system from drying out if it is switched on and in a rest period for a long time, e.g. over night.
- Reset	Resets the parameters to the default values.

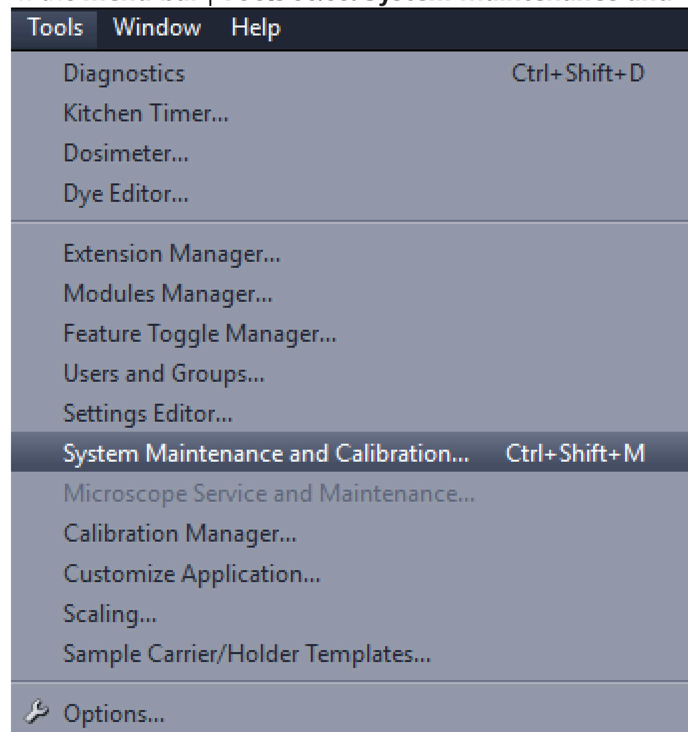
Parameter	Description
Phase Gradient Contrast Settings	
- Automatic Half Pupil Angle Adaptation	A phase gradient contrast image is calculated from two single images acquired with two different angles of the half pupil. If activated, the optimal choice of the angles is calculated depending on the current X/Y Position insides the well.

14.3.12 Performing a Celldiscoverer calibration

The Celldiscoverer calibration wizard is used for hardware calibration of the system. The individual components can be selected for re-calibration. Calibration data are stored for comparison.

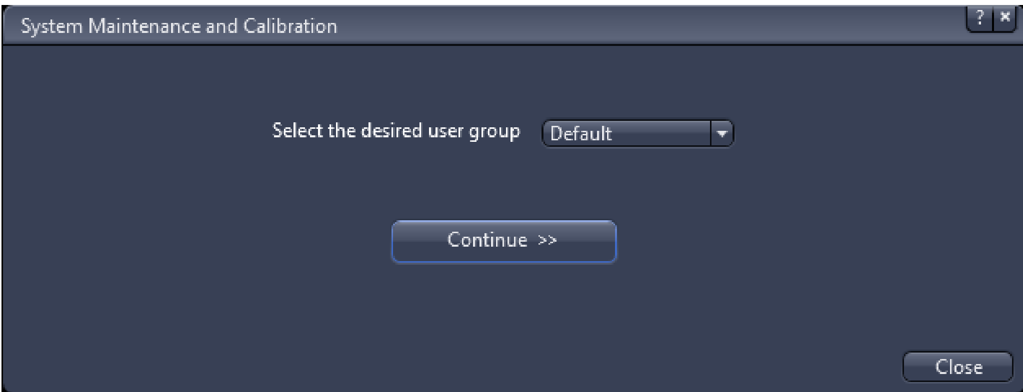
If you want to perform a calibration using the wizard, the following steps are necessary.

1. In the **menu bar** | **Tools** select **System Maintenance and Calibration...**

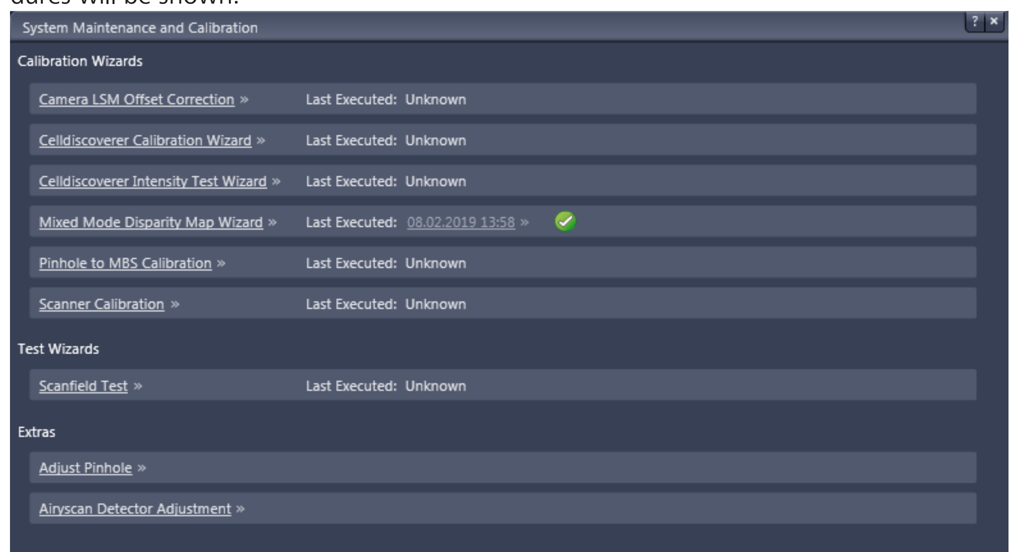


→ The **System Maintenance and Calibration** dialog opens.

2. Click on **Continue >>**.

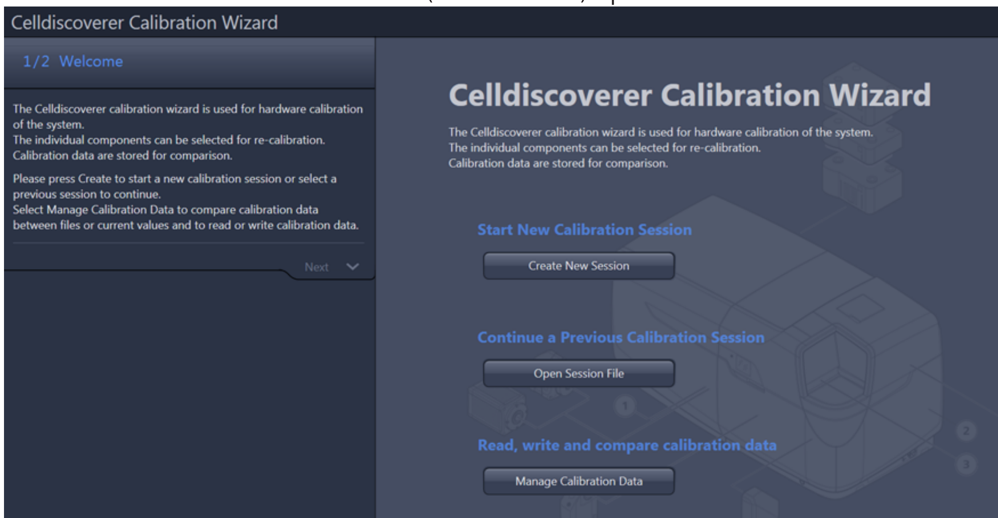


→ In the **System Maintenance and Calibration** dialog, the available Calibration procedures will be shown.



3. Click on **Celldiscoverer Calibration Wizard >>**.

→ The Celldiscoverer Calibration Wizard (1/2 Welcome) opens.



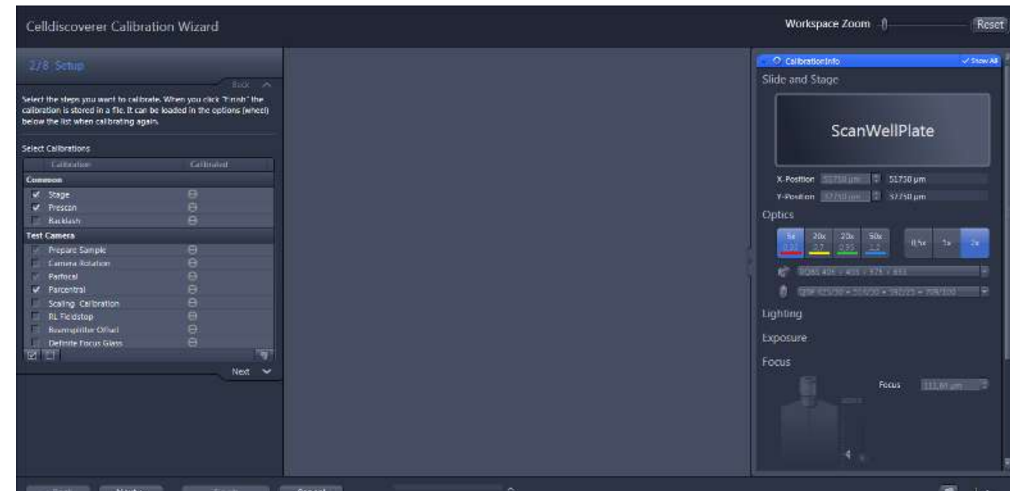
→ Three option are available to continue:
- start a new calibration session by clicking on **Create New Session**
- continue with a previous calibration session by clicking on **Open Session File**
- read, write and compare calibration data by clicking on **Manage Calibration Data**

4. To perform a new Celldiscoverer calibration, click on **Create New Session**.

→ Additionally, an edit field with a proposed session name and the **Exclude Immersions Objectives** checkbox are hidden.

5. If required, edit the name of the calibration session and activate the **Exclude Immersions Objectives** checkbox.
6. To start the calibration procedure, click on **Create**.

→ The next screen of the wizard will be shown.



7. Select the steps for the calibration procedure by activating the appropriate checkboxes.
8. Click on **Next >** to run the calibration.
9. After the calibration is done, click **Finish**.
   → The calibration results will be stored in a file and the **System Maintenance and Calibration** dialog appears again. The **Celldiscoverer Calibration Wizard >>** item is marked by a green checkmark.
10. Close the **System Maintenance and Calibration** dialog with **Close**.

If the window closes, you have successfully performed the Celldiscoverer calibration.

14.3.13 Mixed Mode Settings

The Celldiscoverer 7 allows the combined acquisition of camera and LSM tracks. This unique **Mixed Mode Acquisition** ensures the precise overlay of the widefield and confocal/Airyscan images. The resulting file contains image(s) of all aligned channels, e.g. for seamless analysis workflows.

In the **Mixed Mode Settings** of the **Acquisition Mode** window, a pixelwise overlay of the widefield and confocal frames is set. Optimal image alignment is achieved by registering the LSM image to the camera image. Therefore, a so-called disparity map is automatically created for each Celldiscoverer 7 system using the *Mixed Mode Disparity Map wizard [▸ 1037]*. As a result, four different image processing functions are automatically carried out when the mixed mode acquisition is activated to correct for variations in scaling (related to total magnification and pixel size), distortion, position/offset, and pixel numbers (cropping of the image).

See also

- Parameters for LSM Imaging Modes [▸ 688]
- Acquisition Mode Tool [▸ 687]

14.3.13.1 Camera + LSM Combined

By clicking on **Combined**, the direct overlay of WF and confocal frames is automatically adapted to the tracks for all zooms and magnifications. For the combined acquisition of WF and confocal tracks in the mixed mode, no scan field offset and rotation is possible and the bit depth is fixed to 16 bit per pixel. The mixed mode is available for **Widefield**, **LSM confocal**, **Airyscan HS**, and **Airyscan MPLX** tracks. The following tracks can be combined:

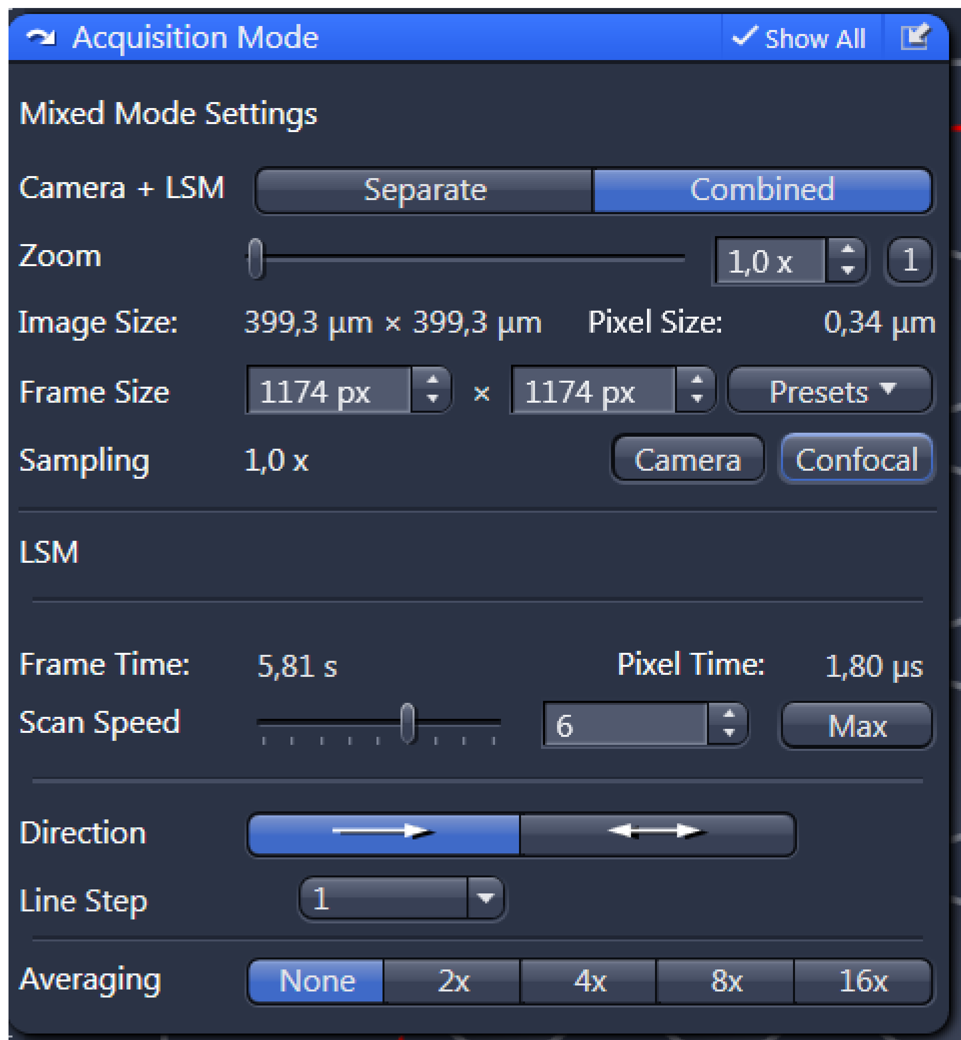
- Widefield + LSM confocal
- Widefield + LSM confocal + Airyscan HS
- Widefield + Airyscan HS
- Widefield + Airyscan MPLX HS

The WF track is automatically set as the first track.

Aquiring z-stack images:

Z-stack acquisition in mixed mode will automatically activate **Full z-stack per Channel**. In the experiment, channel 1 is acquired for all z levels before channel 2 is acquired.

Mixed Mode Settings with **Widefield** and **LSM confocal** track:



Zoom: Zoom is automatically adapted to both camera and LSM track. The widefield image is interpolated to correspond to the LSM image. Zoom is affecting image size and sampling. The number of pixels (frame size) is constant when changing the zoom.

Frame size: is adapted according to the active sampling. Changes of the frame sizes (e.g. via Presets) influence the sampling.

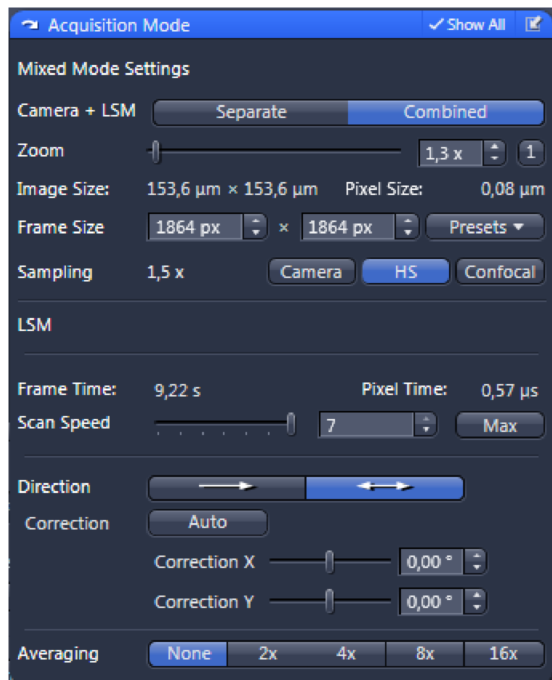
Sampling: The channel with highest demand for sampling is master and defines the default.

- **Confocal**: xy sampling set for 1.0 x Nyquist (2 times sampling) to achieve optimal settings for confocal resolution. The camera frame is cropped to the LSM frame.
- **Camera**: Adjusts the LSM parameters to match the physical pixel size of the camera.

LSM specific settings (for more information, see *Parameters for LSM Imaging Modes [▸ 688]*):

- **Scan Speed**
- **Direction** Monodirectional or Bidirectional
- **Line Step**
- **Averaging**

Mixed Mode Setting with Widefield and Airyscan HS/ Airyscan MPLX HS track:



Sampling: The channel with highest demand for sampling is master and defines the default.

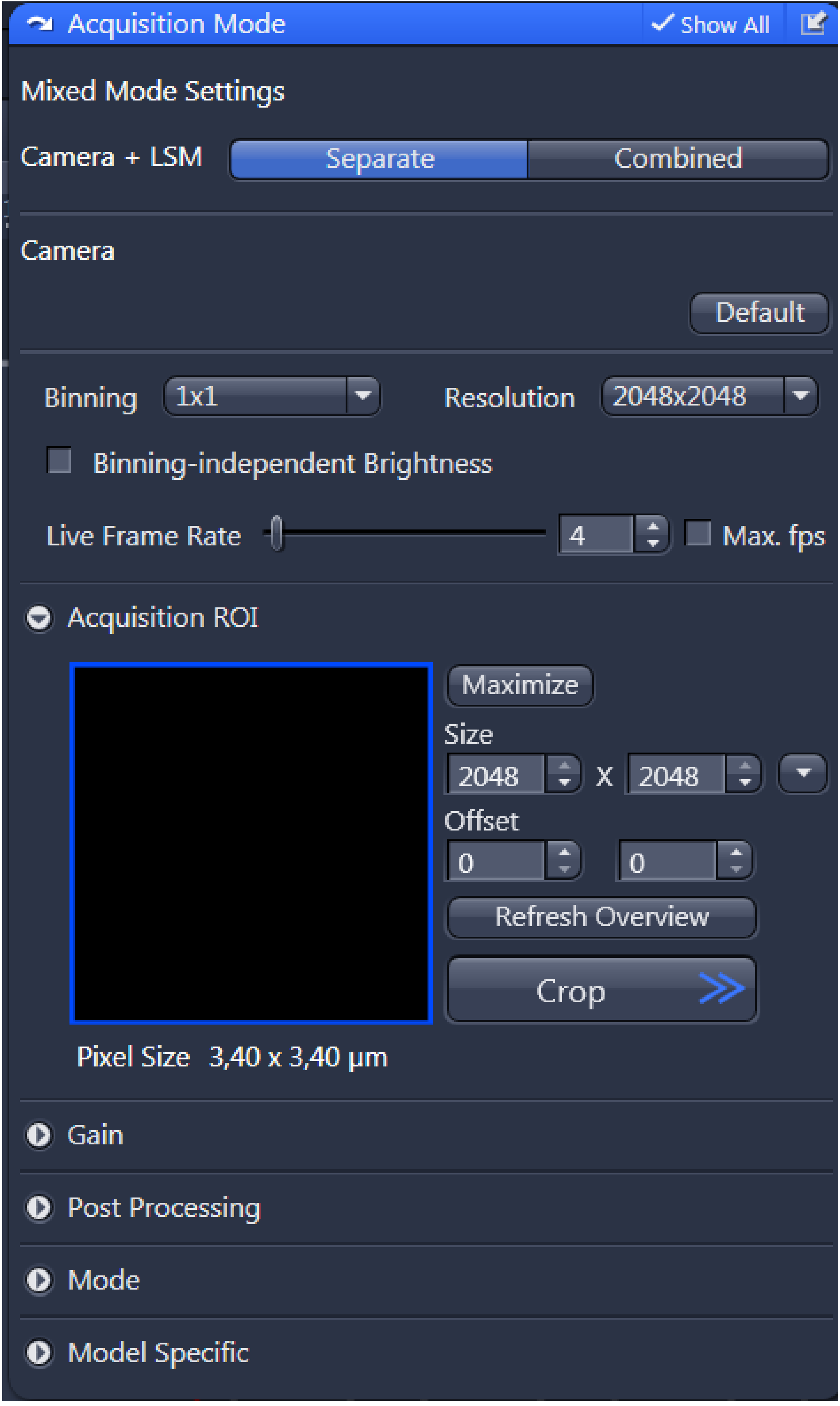
- **Confocal**: xy sampling set for 1.0 x Nyquist (2 times sampling) to achieve optimal settings for confocal resolution. The camera frame is cropped and resampled to match the LSM frame.
- **HS/ MPLX HS**: xy sampling set for 1.5 x Nyquist (3 times sampling) to achieve better SNR. The camera frame is cropped and resampled to match the LSM frame.
- **Camera**: Adjusts the LSM parameters to match the physical pixel size of the camera.

14.3.13.2 Camera + LSM Separate

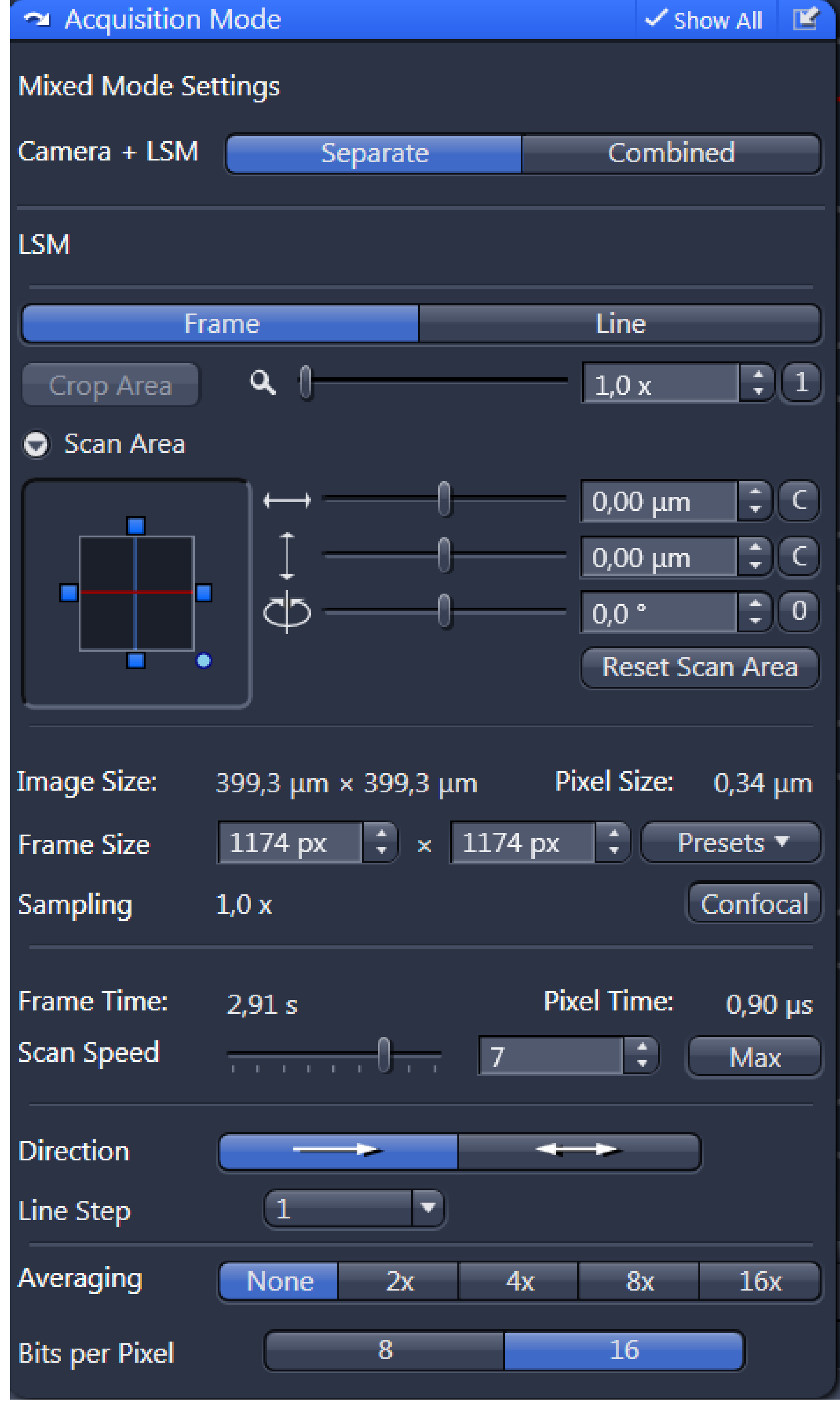
For separate acquisition of WF and confocal tracks, press the Separate button in the **Mixed Mode Settings**. The frame size of the camera is reset to maximum. The frame size of the LSM track is not changed.

Depending on the selected track in the channels window, the **Acquisition Mode** tool is adapted, when the **Separate** tab in the **Mixed Mode Settings** is active.

For an active WF track, all camera relevant parameters are shown (for details see the chapter for *Acquisition Mode [687]*). The frame size of the camera is reset to **Maximize**. Other parameters are unaffected.



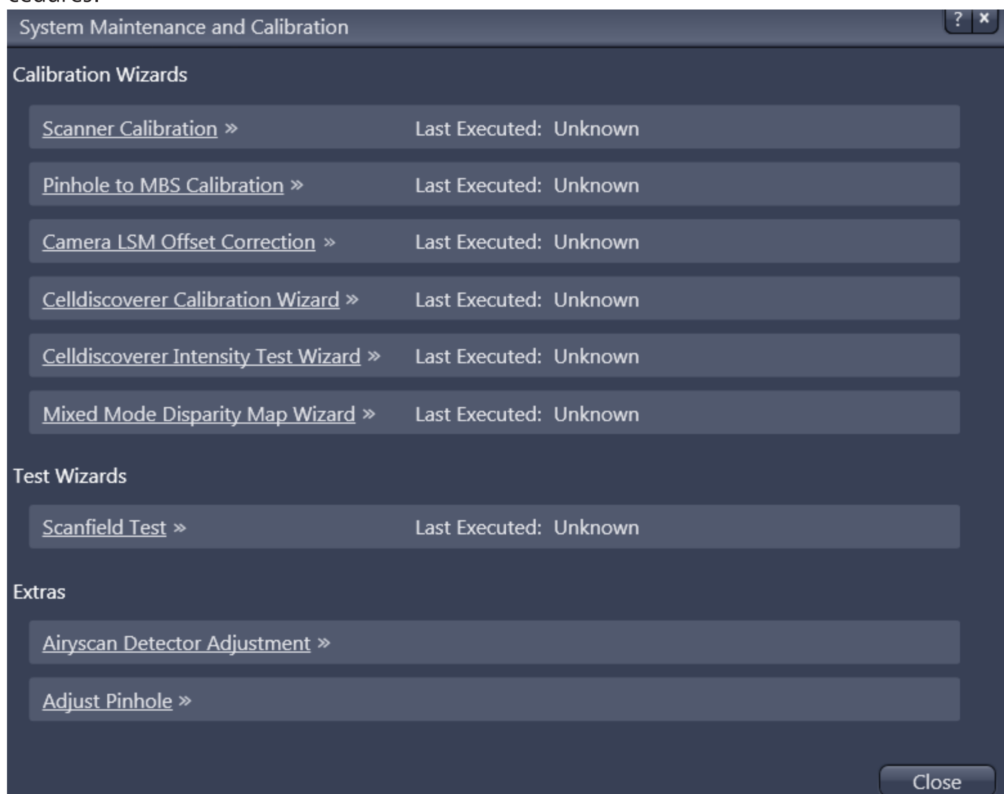
For an active LSM track, all relevant scanning parameters are shown.



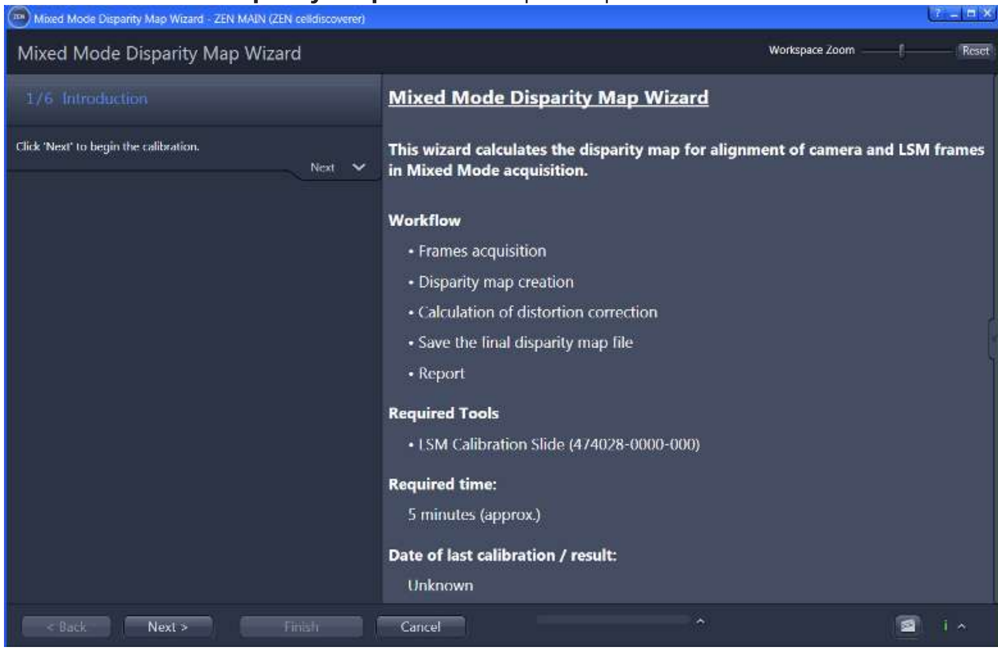
14.3.14 Calculating the disparity map for mixed mode acquisition

The wizard calculates the disparity map for alignment of camera and LSM frames in mixed mode acquisition. Based on a reference image which is acquired in the wizard, the disparity map (an image processing function) is generated that is applied for all LSM frames to ensure a pixelwise overlay with the WF frames in the mixed mode acquisition.

1. In **Tools** select **System Maintenance and Calibration...**
   → The **System Maintenance and Calibration** dialog opens.
2. Click on **Continue**.
   → The **System Maintenance and Calibration** dialog shows the available calibration procedures.



3. Click on **Mixed Mode Disparity Map Wizard**.
   → The **Mixed Mode Disparity Map Wizard** Step 1/6 opens.

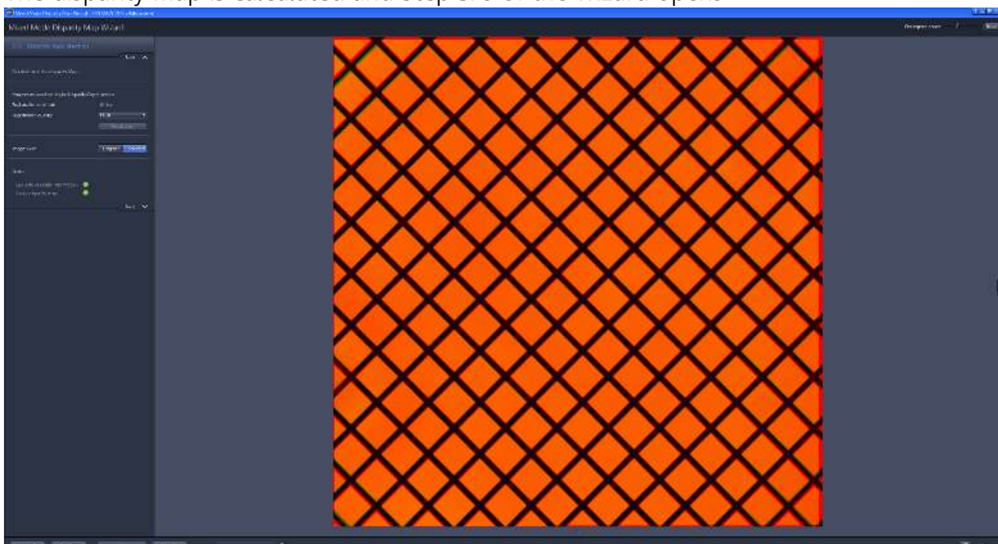


4. Click on **Next**.

→ Step 2/6 of the wizard opens.

5. Load the calibration slide in the center slide position (3-slide holder) and click **Load Sample**
   Calibration Info Tab: Display of acquisition parameters automatically set in the wizard.
   ■ **Optics**: Magnification used to acquire the reference image.
   ■ **Lighting**: WF track is acquired using transmitted light. Confocal track is acquired with the 640 nm laser.
   ■ **Exposure**: Exposure time of camera.
   ■ **Focus**: Focus position (z). The stage position and focus is set automatically. The correct focus position can also be defined via **Auto-Focus**. The software autofocus settings can be set in the **Software Autofocus** tab.
   Acquire the WF and LSM (confocal) image:
   ■ Activate **Auto-Focus** to find the correct focus position. Otherwise, select **Already in focus**.
6. Click on **Image acquisition**.
7. Click on **Next**.

→ The disparity map is calculated and Step 3/6 of the wizard opens.



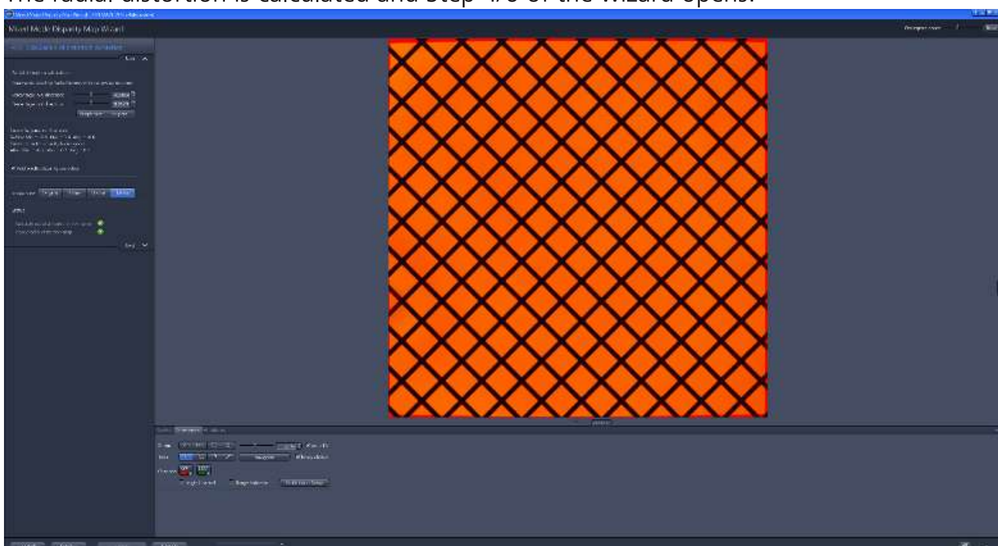
Parameters used by the RegionDisparityMap function:
- **Registration Method**: Affine.
- **Registration Quality**: High (default). If the result is not accepted and the channels do not overlay correctly, activate the drop down menu to select between low, medium, high and highest and press recalculate.

Image View:
- Original: Displays the overlay of WF and confocal frame without applying the disparity map.
- Corrected: Displays the corrected overlay of WF and confocal frame.

Status:
- Successful calculation of disparity information is indicated by a green check mark.
- Successful application of the disparity map is indicated by a green check mark.

8. After successful calculation, click on **Next**.

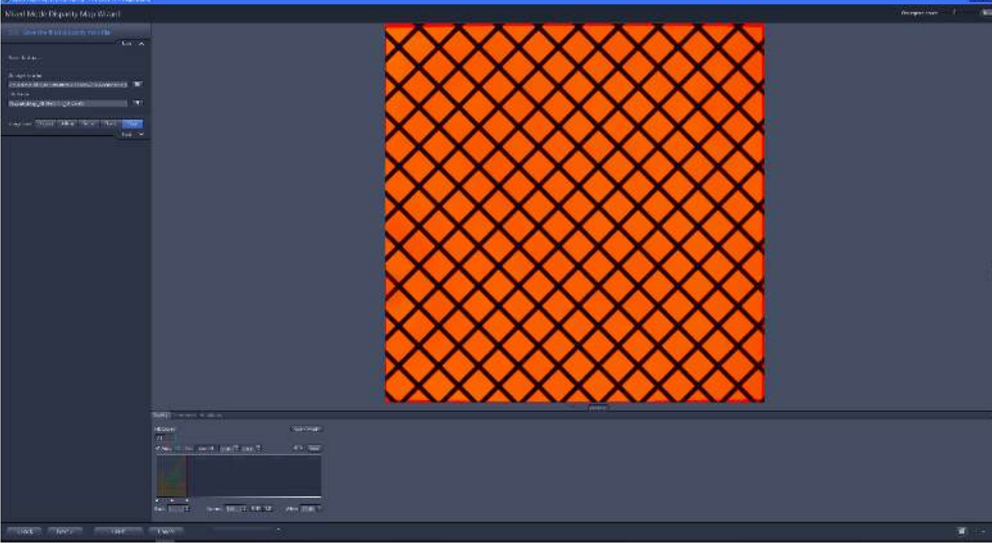
→ The radial distortion is calculated and Step 4/6 of the wizard opens.



Elastic Registration Statistics: A good result is achieved when the average is in the range of 1 pixel.
Image View:
■ Original: Displays the overlay of WF and confocal frame without applying the radial distortion correction.
■ Affine/Distort/Elastic: Display of the individual correction steps.
Status:
■ Successful calculation of radial distortion information is indicated by a green check mark.
■ Successful application of the radial distortion map is indicated by a green check mark.

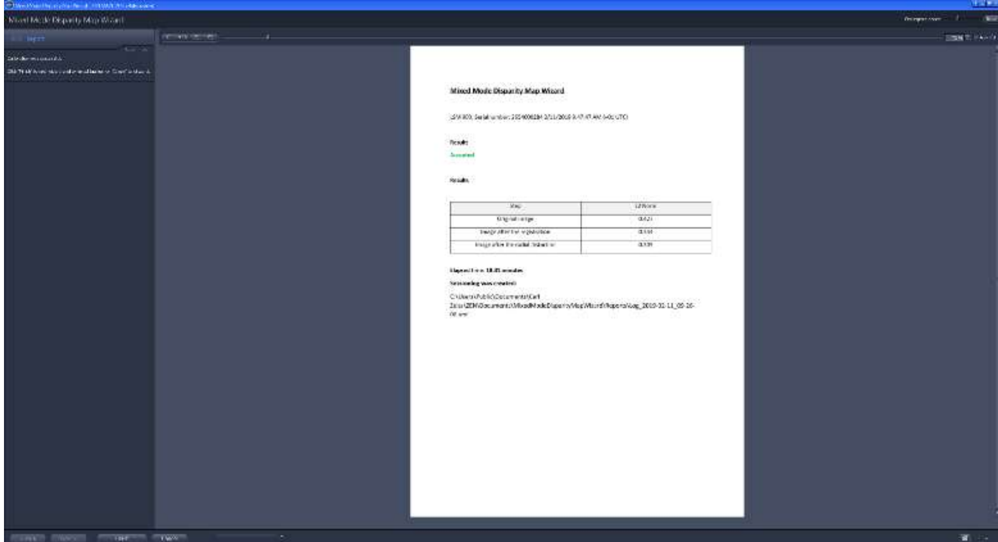
9. After successful calculation, click on **Next**.

→ The final disparity map image is saved and Step 5/6 of the wizard opens.



Storage Location: Define the folder (C:\Users\Public\Documents\Carl Zeiss\ZEN\Documents\MixedModeDisparityMapWizard).
File Name: Define the file name (default: DisparityMap_Date_Time).
Image View:
■ Original: Displays the overlay of WF and confocal frame without applying the radial distortion correction.
■ Affine/Distort/Elastic: Display of the individual correction steps.
■ Final: Displays the final image based on the corrections.

10. Click on **Next**.
    → Step 6/6 of the wizard opens.



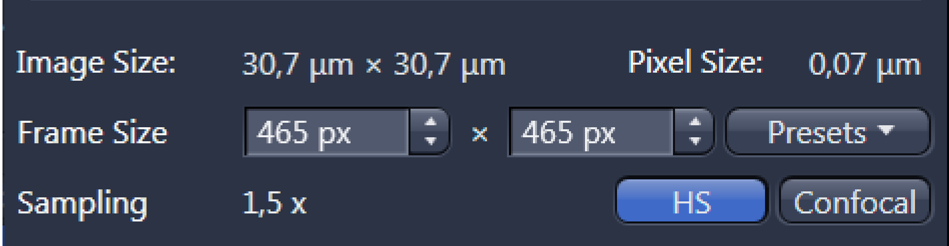
- **Finish**: Ends the wizard and writes the calibration.
- **Cancel**: Discards the calibration.

11. Click on **Finish**.

The wizard is closed and the successful calculation of the disparity map is indicated by a green check mark.

14.3.15 Airyscan HS

The Celldiscoverer 7 contains the specific **Airyscan HS** (high sensitivity) mode.



On the **Acquisition Mode** tool, the **Airyscan HS** specific parameter options are the following:

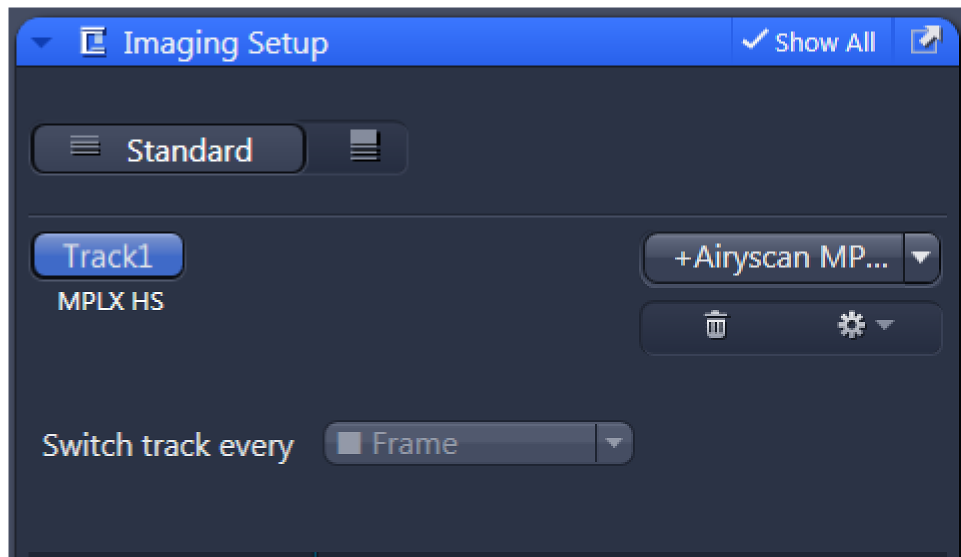
Parameter	Description
Sampling	
– HS	XY sampling set for 1.5x Nyquist to achieve better SNR.
– Confocal	XY sampling set for 1.0x Nyquist to achieve confocal resolution with increased signal-to-noise with Airyscan detector.

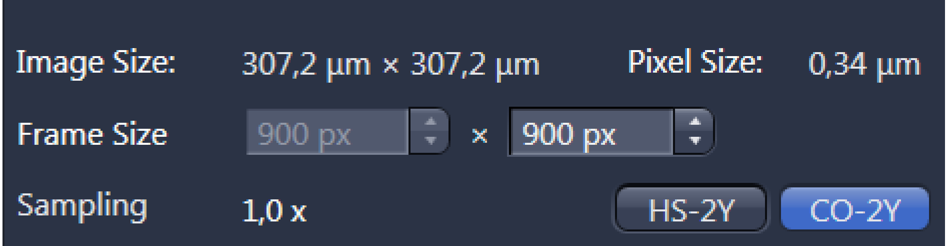
For information about the general parameter options of the **Acquisition Mode**, see *Acquisition Mode Tool [▸ 687]*.

14.3.16 Airyscan MPLX HS

The Celldiscoverer 7 contains the specific **Airyscan MPLX HS** (high sensitivity) mode. This track type cannot be combined with **Confocal**, **HS** or **WF** tracks. **Mixed Mode Acquisition** is not possible.

The **Airyscan MPLX HS** track is a selection option in the **Imaging Setup**.





On the **Acquisition Mode** tool, the **Airyscan MPLX HS** specific parameter options are the following:

Parameter	Description
Sampling	
– HS-2Y	XY sampling set for 1.5x Nyquist to achieve better SNR.
– CO-2Y	XY sampling set for 1.0x Nyquist to achieve confocal resolution with increased signal-to-noise with Airyscan detector.

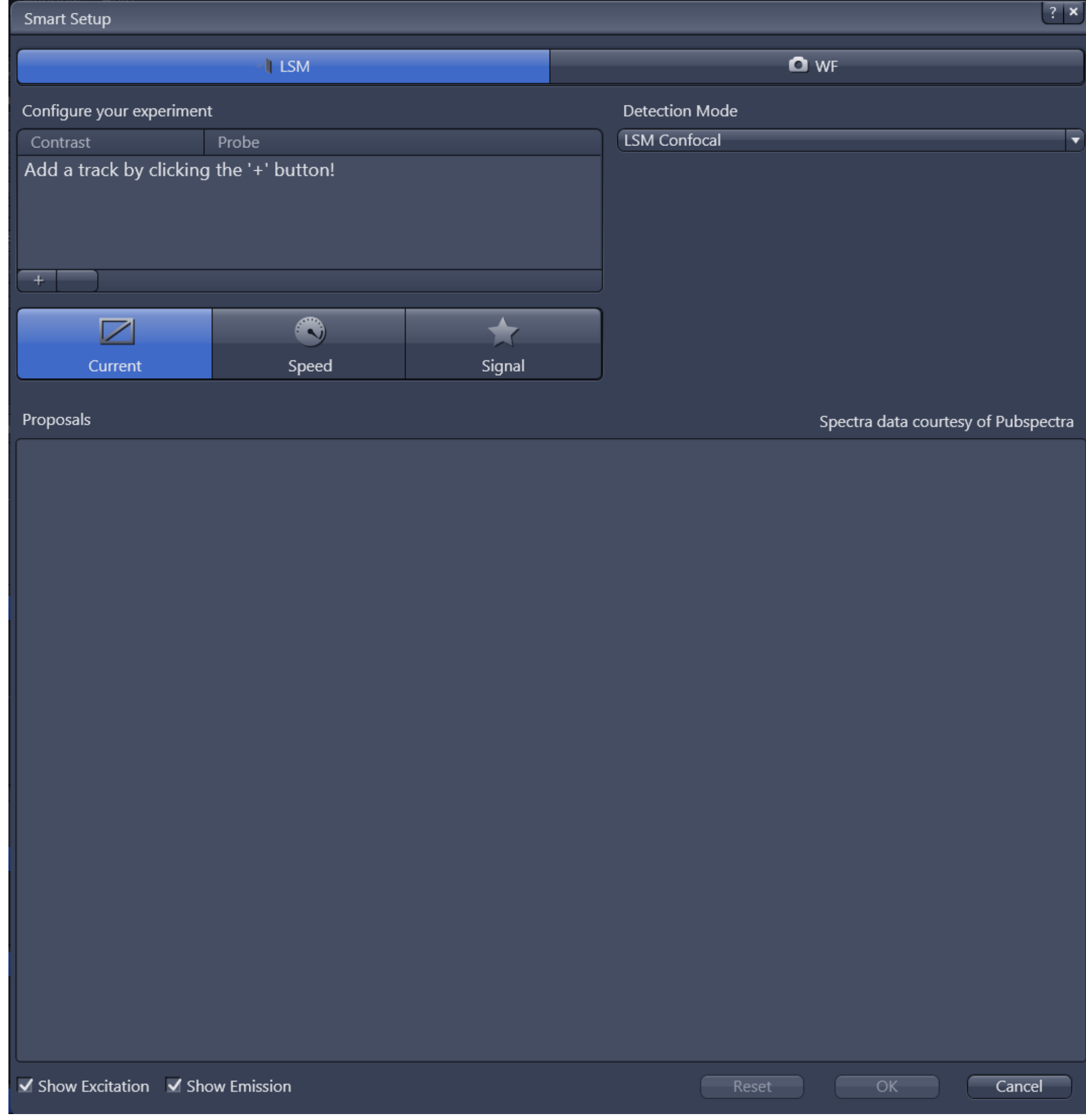
For information about the general parameter options of the **Acquisition Mode**, see *Acquisition Mode Tool [▸ 687]*.

See also

Acquiring LSM 900 images with Airyscan 2 multiplex modes [▸ 76]
Acquiring LSM 980 images with Airyscan 2 multiplex modes [▸ 79]

14.3.17 Smart Setup

Airyscan HS and **Airyscan MPLX HS** tracks are selected as a detection mode in the drop down menu of the LSM tab.

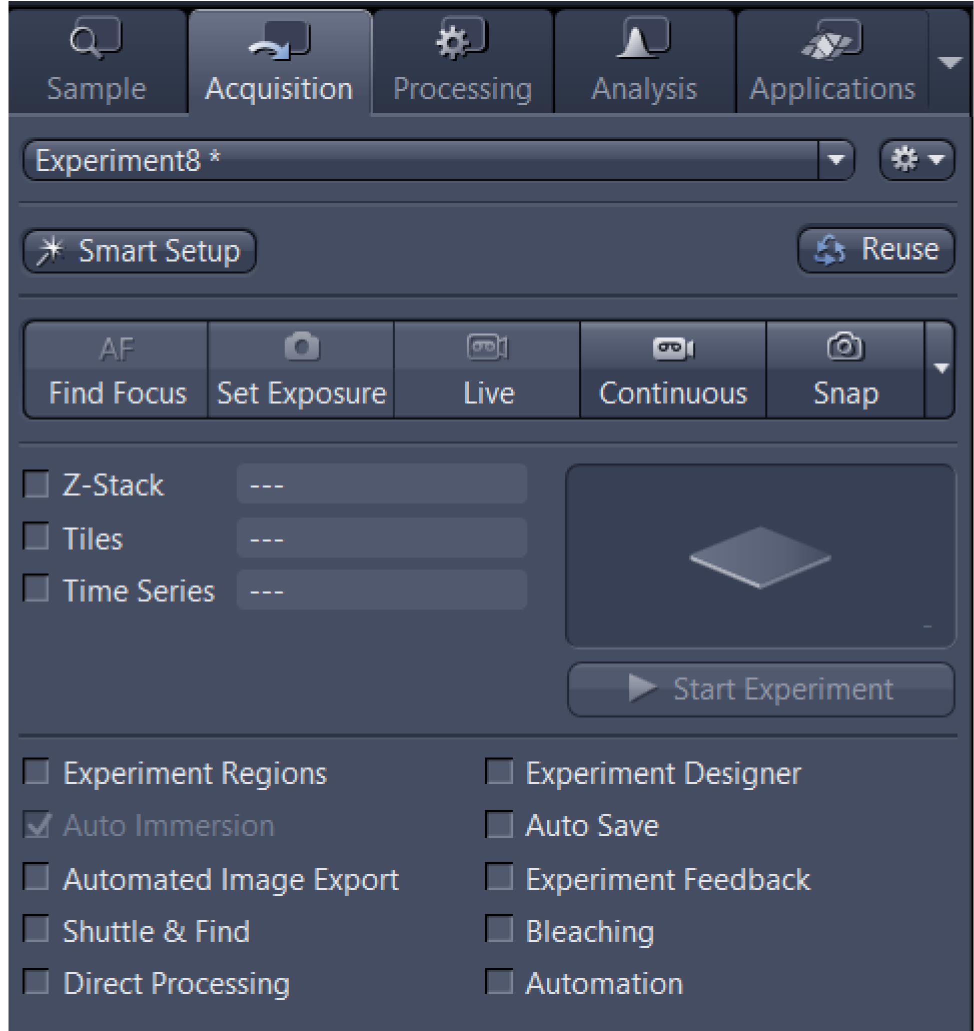


14.3.18 Auto Immersion for Celldiscoverer

14.3.18.1 Introduction

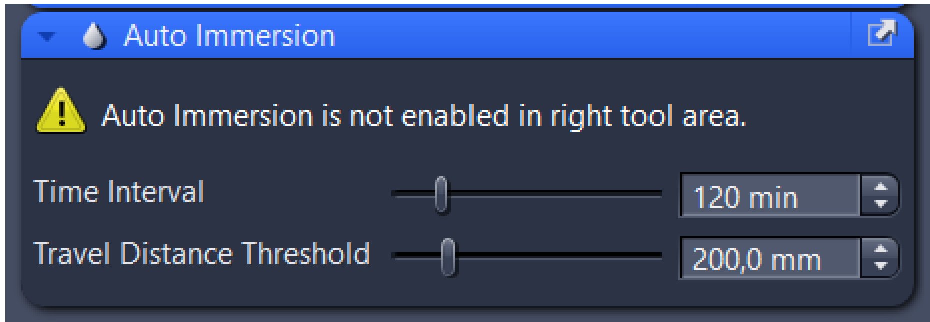
The **Auto Immersion** functionality in ZEN (blue edition) is used for adding immersion fluid (water) to water immersion objectives and automatically renewing the immersion fluid during experiments.

Working with Auto Immersion in ZEN is easy. Firstly, create the immersion or renew it, when the immersion fluid starts to dry out. This is performed using the **Auto Immersion** tool in the **Right Tool Area**. For Celldiscoverer 7 containing the 50x/1.2 water immersion objective, the **Auto Immersion** is activated automatically.



You can also configure Auto Immersion functionality for experiments. This can be done in the **Auto Immersion** tool in the **Left Tool Area** on **Acquisition** tab. E.g. for time series experiments you can set a time interval after which the created immersion will be renewed automatically during the experiment. Please ensure to enable immersion in the **Auto Immersion** tool in the **Right**

Tool Area. Otherwise, the following warning message is shown: “Auto Immersion is not enabled in the right tool area”.



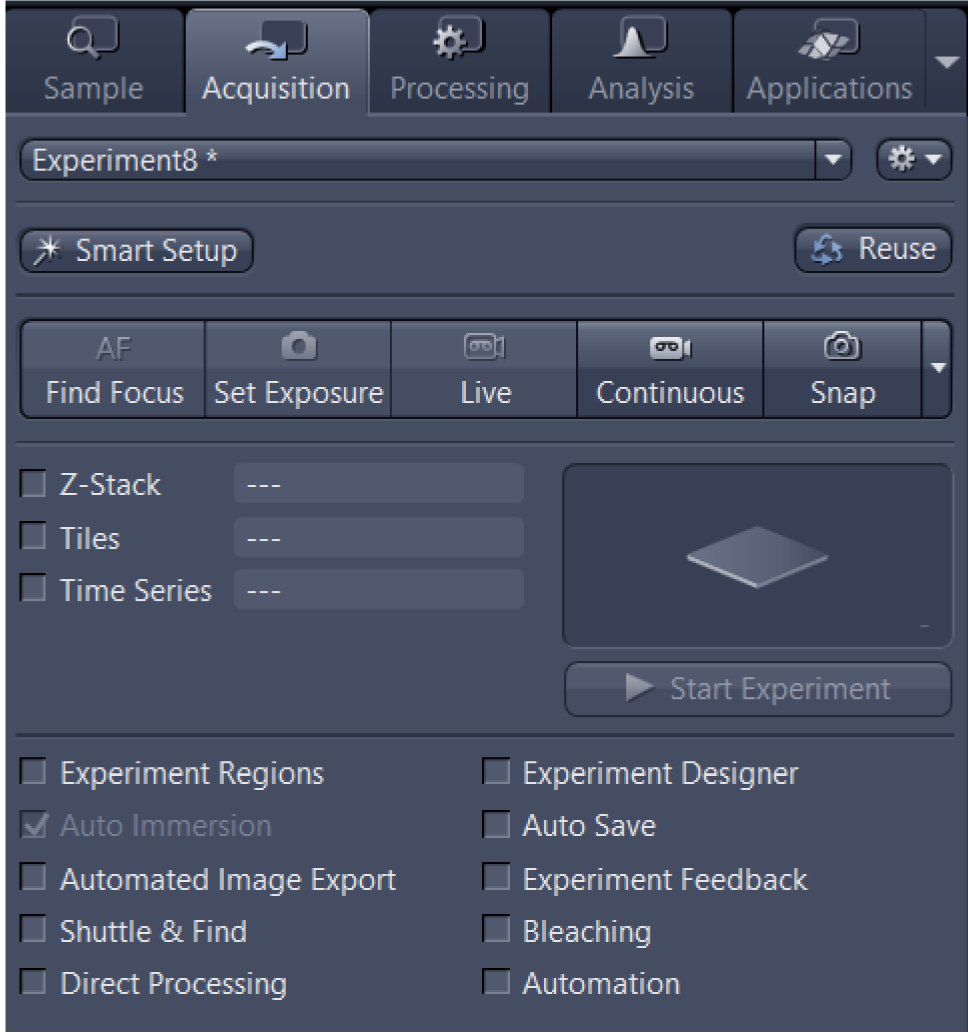
In the following chapters we describe step-by step how you prepare working with Auto Immersion objectives and how you can use Auto Immersion for experiments.

See also

Preparing Auto Immersion [▷ 1046]
Using Auto Immersion for an experiment [▷ 1047]

14.3.18.2 Preparing Auto Immersion

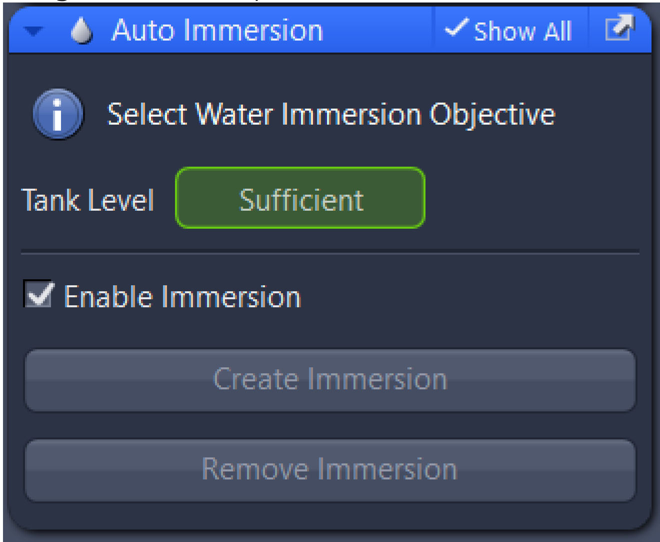
For Celldiscoverer 7 containing the 50x/1.2 water immersion objective, the Auto Immersion is activated automatically.



When using the water immersion objective for imaging, the creation of the water immersion must be enabled.

The following steps have to be performed:

1. In **Right Tool Area**, open the **Auto Immersion** tool and activate **Show All**.



2. Activate the **Enable Immersion** checkbox to ensure auto immersion functionality.
   → The **Create Immersion** and **Remove Immersion** buttons will be available.
3. Select the water immersion objective.

The immersion process can be controlled manually using the tool in the **Right Tool Area**:

- Click on **Create Immersion**. This initiates the creation of one immersion procedure.
- Click on **Remove Immersion**. This initiates the removing of the immersion fluid.

The immersion creation or removing procedures are independent from the configured settings in the **Auto Immersion Refill Options**, see chapter *Celldiscoverer Options [▸ 1029]*.

14.3.18.3 Using Auto Immersion for an experiment

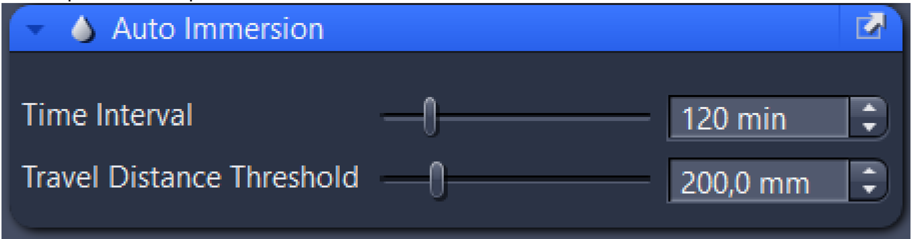
If you want to use Auto Immersion during an experiment, the following steps are necessary.

Prerequisite

- ✓ You have set up an experiment (e.g. a time series experiment).
- ✓ You have read the chapter *Preparing Auto Immersion [▸ 1046]*. Basically you have to perform the steps described in this chapter before each experiment.
- ✓ You can modify the Auto Immersion Refill Options (not recommended) , read chapter *Celldiscoverer Options [▸ 1029]*.

1. Switch to the water immersion objective you want to use in the experiment. The initial immersion will be created automatically.
2. Select **Acquisition** tab.

3. In the **Auto Immersion** tool in the **Left Tool Area**, you can configure the parameters for **Time Interval** and/or **Travel Distance** for the experiment. That event which will take place first will activate the renewing of the immersion during the experiment. For time series experiments we can recommend time intervals for up to 120 minutes, if necessary. For tiles/ multi-position experiments we recommend the re-immersion after 200 mm travel distance.



You have successfully configured auto immersion for an experiment.

14.3.18.4 Auto Immersion Tool

The Auto Immersion tool in the **Right Tool Area** in general is used to check the immersion status and to operate the immersion functions.

Parameter	Description
Tank Level	Shows the current filling level of the immersion fluid tank.
Enable Immersion	Must be activated. Displays the controls for immersion.
Create Immersion	If you click on this button, the system will automatically add the immersion fluid to the selected immersion objective. If the fluid was added once, the button is grayed out.
Remove Immersion	If you click on this button, the system will automatically remove the immersion fluid from the selected immersion objective.

14.3.18.5 Auto Immersion Tool

The Auto Immersion tool in the **Left Tool Area** | **Acquisition** tab is used for experiments only. You can configure the time interval and travel distance for automatic immersion events in here.

Parameter	Description
Time Interval	Here you can adjust the time interval for auto immersion. E.g if you set the value to 30 min, the immersion is automatically renewed every 30 minutes during a running experiment.
Travel Distance	Here you can adjust the travel distance for auto immersion. E.g. if you set the value to 80 mm, the immersion is automatically renewed after the stage has moved a distance of 80 mm.

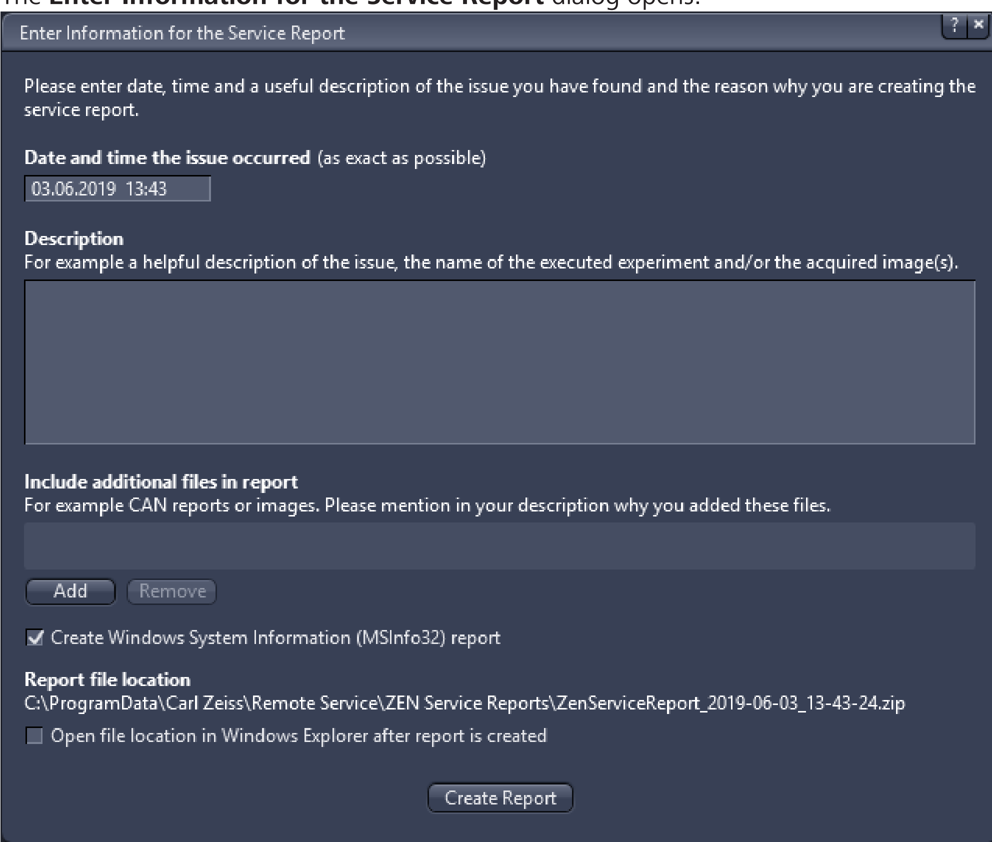
15 Service / Maintenance

15.1 Creating a Service Report

The service report contains log files from MTB and ZEN.

If you want to create a Service Report, the following steps are necessary.

1. In the **menu bar** click on **Help > Create Service Report...**
   → The **Enter Information for the Service Report** dialog opens.



2. Enter date, time, and an useful description of the issue occurred and the reason for creating this service report.
3. To include additional files to the report, click **Add**.
   → The Windows Explorer opens.
4. Open the folder with the file to be added.
5. Select the file.
6. Click on **Open**.
   → The Windows Explorer closes. Location and file name of the added file are shown in the display field.
7. To remove a file from the display field again, select it and click **Remove**.
8. If required, activate **Open file location in Windows Explorer after report is created**.
9. Click **Create report**.

The **System information** window appears, showing the storage progress.

If the window closes, you have successfully created the Service Report.
This process might take several minutes depending on the system, size of the logs and if the Windows system information is collected too. The storage location of the Service Report is C:/ProgramData/Carl Zeiss/Remote Service.

16 FAQ

16.1 What can I do If my image is too dark?

Try to increase the exposure time by performing the following settings:

- in the **Locate** tab click on the **Set Exposure** button. This will calculated the correct exposure time automatically.
- in the **Camera** tool manually adjust the **Time** slider until you achieve the desired result.
- in the **General View Options > Display tab** adjust the display curve, see chapter *Adjusting Live Image Settings [▸ 30]*.

16.2 How can I balance my images color?

To perform an automatic or manual white balance you must use a color camera. In the **Left Tool Area > Locate tab > Camera tool** the **White Balance** section appears. There you can perform a white balance with one of these methods:

Auto Method:

1. Move the sample out of the **Live** window's field of view, so that you only see the background (essentially the light source).
2. Click on the **Auto** button.
3. The white balance will be calculated automatically. Afterwards move your specimen back into the field of view.

Interactive/Pick… Method:

1. Click on the **Pick…** button.
2. Click on a white area of the **Live** window which should be represented as white.

This area will be used as a reference for the white balance.

3200K Method:

Use this method if working with a halogen bulb.

1. If available set the light source to 3200K by pressing the **3200K** button located on the body of the microscope.
2. Click on the **3200K** button.

This method is also largely depending on the quality/age of your bulb. If the color rendition is not as desired, try the **Auto** or the **Interactive/Pick**… methods above.

5500K Method:

Use this method if working with a LED.

1. Click on the **5500K** button**.**
2. If the color rendition is not as desired, try the **Auto** or the **Interactive/Pick**… methods above.

If none of the above methods produce a satisfactory result, one can additionally manually adjust the **Color Temperature** slider.

16.3 How can it be that my image has dust or a shadow, although my specimen is clean?

If the dust is not on your specimen, then the best method is to clean the optical elements that lay in the imaging pathway of your microscope. However if that poses a problem, alternatively, you can perform a Shading Correction as shown below. This solution has some limitations, especially if the dust is very dark or thick.

1. Move your sample out of the Field of View until you see nothing but the light source/dust.
2. In the **Camera** tool, in the **Post Processing** section, click on the **Channel Specific** button.
3. Move your sample back into the Field of View.

16.4 Why my image seems to look that something have burned in? (i.e. a shadow of a previous specimen?)

Check that The Shading Correction of the previous experiment is **not** already adjusted.

1. In the **Camera** tool open the **Post Processing** section
2. Deactivate the **Shading Correction** checkbox.

16.5 How can I fix a color gradient cast?

To fix a color gradient cast, you can try the following:

1. Move your sample out of the Field of View until you see nothing but the light source.
2. In the **Camera** tool, in the **Post Processing** section, click on the **Channel Specific** button.
3. Move your sample back into the Field of View.
4. Perform a **White Balance**, see chapter *How can I balance my images color? [▸ 1050]*

See also

- Why my live image shows extreme colors in comparison to what I see in the eyepieces? [▸ 1052]

16.6 What can I do if my live image is of a low quality and looks pixelated?

1. In the **Camera** tool > **Mode** section in the **Live Speed** dropdown list select the entry **Slow**.
2. Right-click on the live image and select the **Fit to View** entry.
3. Optionally, you can also , in the **Dimensions** tab, activate the **Interpolation** checkbox.

16.7 What can I do if my live image is slow?

Solution A

The live speed of your image is possibly set too slow. Increase the Live Speed.

1. In the **Camera** tool in the **Mode** section, select a faster speed from the **Live Speed** drop-down menu. There are at most three choices, depending on the camera: **Slow**, **Medium**, **Fast**.

Solution B

The exposure time is possibly set to high, respectively improper. Optimize your settings in the **Camera** tool, in the **Exposure time** section.

1. On the **Locate** tab click on the **Set Exposure** button.
2. Alternatively in the **Camera** tool click on the **Set Exposure** button.
3. Manually adjust the **Time** slider until you achieve the desired result.

16.8 What can I do if my live image is mostly red/blue?

Check whether the checkbox **Range Indicator** is activated. If this is the case, the display switches to the **Single Channel** mode. The channel will be displayed monochrome. Simultaneously you see areas where the camera sensor is saturated, shown in red. Areas in which the pixel values = 0, are shown in blue. If this is not needed anymore deactivate the checkbox.

16.9 What can I do if my live image is still black or white after setting the exposure?

Check to see that your display curve is not set all the way to the left/right. Try to reset the display curve by clicking in the **Display** tab on the **Reset** button to achieve the default setting.

16.10 Why my live image shows extreme colors in comparison to what I see in the eyepieces?

The reason could be, that your **display curve** is not adjusted.

1. Open the **Display** tab.
2. Click on the **Reset** button.
3. Click on the **0.45** button or set Gamma 0.45.
4. You can additionally click on **Min/Max** or **Best Fit** button.

See also

Why is my image color not the same that I see through the eye pieces? [1053]

16.11 Why is my image resolution lower than the given camera specification?

Because you chose a wrong or improper setting.

Prerequisite ✓ You are on the **Locate** tab.

1. In the **Camera** tool, in the **AcquisitionROI** section, click on the **Maximize** button.
2. In the **Mode** section check that **Binning** is set to **1x1.**

16.12 What can I do if I do not see a focused live image?

Refocus the specimen on the microscope. You may activate the **Focus Bar** as an additional aid.

1. Open the context menu via right-click in the **Live image.**
2. Select the entry **Focus Bar**.

The **Focus Bar** will be shown within the **Live image** now.

16.13 Why is my image color not the same that I see through the eye pieces?

This is largely dependent on the color of your light source. The following instruction assumes that your light source is set to white.

1. In the **Camera** tool, in the **Settings** section click on the **Default** button, to set the camera back to factory default.
2. Click on the **Set Exposure** button.
3. In the **Display** tab, click the **0.45** button. If you do not see this button, activate the **Show All** mode.

See also

How can I balance my images color? [1050]

Glossary

Airyscan Principle

A classic confocal microscope illuminates one spot on your sample to detect the emitted fluorescence signal. Out-of-focus emission light is rejected at a pinhole, the size of which determines how much of the airy pattern reaches the detector. You can increase the resolution by making the pinhole smaller, but signal-to-noise drops significantly since less valuable emission light is passing through. With Airyscan ZEISS introduces a new concept. Instead of throwing light away at the pinhole, a 32 channel area detector collects all light of an Airy pattern simultaneously. Each detector element functions as a single, very small pinhole. Knowing the beampath and the spatial distribution of each Airy pattern enables a very light efficient imaging: you can now use all of the photons that your objective collected.

ApoTome - Global bleaching correction

For each raw image the total (sum) intensity is measured and a global decay curve determined. This decay factor is used to correct the brightness of all pixels in the raw image. This method is suitable for samples with only one fluorescent dye present.

ApoTome - Local bleaching correction

If more than one fluorescent dye is present in the sample, a single decay curve cannot be used to correct bleaching. This is the case for most biological samples especially when considering the contribution of autofluorescent substances to the total signal detected. The solution is to use a local bleaching correction determining a decay factor for each individual pixel position in each raw image which effectivel removes artifacts also for complex dye combinations.

ApoTome Bleaching correction

The main reason for residual stripe artifacts in ApoTome images is the fact, that acquiring at least 3 grid images for one resulting processed image leads to bleaching of the fluorescent dyes. This bleaching leads to brightness differences in the raw images and to artifacts when processing the data uncorrected. The principle for correcting bleaching in widefield data is the fact, that no matter, how far away from the focal plane the detector is placed, the sum intensity emitted from the sample remains the same. This is also true for the grid images. This fact is being used for the ApoTome. Two methods exist, both of which are patented.

Binning

Binning is understood to mean the combination of neighboring image elements (pixels) on the image sensor itself, e.g., the CCD sensor in a digital camera. Source: Wikipedia

Bleaching Correction

The characteristics of a widefield fluorescence microscope are based on the assumption that all Z-planes have the same total brightness, irrespective of the focus position. Use is made of bleaching correction by applying a correction factor to each Z-plane. However, this assumption does not apply to techniques that result in the generation of optical sections, such as confocal images.

Burst Mode

Burst Mode is some kind of optimization to enable the recording of the fastest framerates, which can be achieved by the used camera hardware. This mode requires some compromises for the sake of speed: it supports only single channel time lapse acquisition, an update of the image display is suppressed while recording and the

maximum time lapse duration is depending from the size of available main memory (minus some space to breathe for the operating system). If a multi channel image needs to be acquired, Burst Mode will be disabled and maximum frame rate can be slower then specified in the camera hardware performance documentation.

Clipping planes

The purpose of clipping planes is to cut open the calculated 3D image so that elements on the inside can be visualized. Clipping planes can cut the volume in such a way that either the front, back or both sides of the volume data are no longer visible. In addition, the clipping plane itself can be given various textures. This is a very important modeling option for analyzing 3D data.

Colocalization

Acquiring fluorescence images in several channels makes it possible to visualize the relationship between biological structures. A combined display of two channels in color overlay mode makes it easier to assert whether the components are "colocalized", i.e. whether they are located at the same position. Conventionally, two fluorescence channels are displayed in the form of a color-coded overlay. The most common form is the red/green overlay. Regions in which both fluorescent dyes are present at the same place are displayed in yellow. It is not possible, however, to make quantitative statements concerning the extent of colocalization on the basis of this display. At best, a qualitative statement is possible with regard to whether or not two dyes are colocalizing. The Colocalization module is able to fill this gap and presents the user with a tool that enables colocalization to be determined quantitatively. Principle: It is always the colocalization of two channels that is analyzed. Colocalization results from the pixel-by-pixel comparison of intensities for each channel.

Constrained Iterative

The best image quality is achieved using the iterative maximum likelihood algorithm (see Schaefer et al.: "Generalized approach for accelerated maximum likelihood based image restoration applied to three-dimensional fluorescence microscopy", J. of Microscopy, Vol. 204, Pt 2, November 2001, pp. 99ff.). This algorithm is able to calculate light from various focal planes back to its place of origin. Consequently, with this method it is possible to derive the 3D structure from fluorescence images with the correct brightness distribution and to visualize optical sections. It is also possible for missing information to be partially restored from neighboring voxels. The spatial resolution can be increased without artifacts up to a theoretical limit (one voxel). It is essential for Z-stacks to have been acquired in accordance with Nyquist. Acquiring sufficient planes above and below the structure of interest is also imperative for achieving good results. As this is a complex mathematical method, the calculation can take longer, depending on the image size and the PC being used.

Costes

Costes et al. (Biophysical Journal, 2004, vol. 86, pp 3993-4003) have published a statistical method with the help of which an attempt is made to determine an optimal colocalization threshold automatically. This takes place by initially maximizing the threshold for both channels and then gradually reducing it. With each step Pearson's Correlation Coefficient is determined for all pixels below the set value. These steps are repeated until the Pearson value is minimized (ideally a value of 0 for perfectly colocalizing channels). See the publication for further details. This method has

been implemented in Colocalization. Clicking on Auto initiates the above iterative process, which, depending on the sample, can take several seconds. The threshold now set corresponds to the confidence criterion calculated. This method works very well with large, diffusely stained structures such as nucleoplasm or diffuse cytoplasmic structures. Under certain circumstances it does not function so well for small structures (e.g. nuclear speckles or vesicular structures), particularly in the case of widefield images, where the signal to background ratio is not as good as it is with methods that involve the generation of optical sections (e.g. LSM, TIRF or ApoTome). The Regions button becomes active as soon as a region is inserted into the scatter plot. It remains active as long as regions are selected or moved there. Activating and deactivating the button makes it possible to switch between threshold selection using the mouse and the selection/moving of selected regions in the scatter plot image. If regions are defined in the scatter plot, the corresponding data appear in the table in addition to the overall image.

Deconvolution

Deconvolution is a method that is used to improve fluorescence images in particular. Image information acquired using a microscope system can never fully reproduce the structures of the actual object. This is because unavoidable distortions occur during acquisition due to the optics and electronics. In addition, particularly in the case of fluorescence microscopes that do not offer any methods for generating optical sections, light from areas of the object outside the objective's focal plane is also always acquired. This covers the structures that the user actually wants to see to a varying degree and therefore leads to a reduction in the contrast and consequently in the visible resolution. These optoelectronic effects can be described mathematically in the form of the point spread function (PSF). If the PSF is known, it is possible to correct the negative effects to a large extent using deconvolution. This produces a completely sharp image of the object that is richer in contrast. Deconvolution is usually performed on Z-stacks, i.e. it is used as a 3D method. However, it can also be used to a limited extent to improve 2D images. A good review of deconvolution can be found in Wallace et al., 2001: A Workingperson's guide to deconvolution in light microscopy; Biotechniques 31: 1076-1097.

Discrete Fourier Transform

The Discrete Fourier Transform functionality is based on the publication: "Multiple imaging axis microscopy improves resolution for thick-sample applications", Jim Swoger, Jan Huisken, and Ernst H. K. Stelzer, OPTICS LETTERS / Vol. 28, No. 18 / September 15, 2003

Display characteristic curve

The display characteristic curve allows you to define the range of the gray value histogram of an image that you want to display on the screen. The limit on the left defines the gray value up to which all pixels are displayed as pure black (black value), while the limit on the right defines the gray value from which all pixels are displayed as pure white (white value). The curvature of the curve defines the so-called gamma value.

Drag&Drop

Literally translates to "drag and drop". Does the moving of objects (eg, files, icons, etc.) on the screen as from one folder to another. Clicking the object with the left mouse button, holding down these, the object moves with the mouse to the desired location.

Dynamic range

The dynamic range describes the number of brightness gradations that a camera or another detector is able to distinguish. Modern, scientific digital CCD cameras, for example, have a dynamic range of up to 2^16 gray levels. In this case we talk of 16 bit cameras.

Experiment Feedback

Experiment feedback allows the definition of specific rules and actions to be performed during an experiment. This allows changing the course of the course of an experiment depending on the current system status or the nature of the acquired data on runtime. Moreover, it is possible to integrate certain tasks like data logging or starting an external application, directly into the imaging experiment. Typically, but not exclusively, such an experiment connects the image pickup with an automatic image analysis.

Fast Iterative

The "Fast Iterative" method is an iterative restoration method that uses only one iteration per convolution step (see Meinel, E. S.: Origins of linear and nonlinear recursive restoration algorithms. J.Opt.Soc.Am, 3 (6), 1986, 787-799). No regularization is used in this case. Due to the fast processing and convergence after just a few iterations, this method is suitable in particular for the processing of larger time lapse images. The results of the method can quickly lead to good results and remove most of the out-of-focus light. They do not, however, create quantitative brightness conditions in the image. If undersampled images are present, artifact formation may also result.

FCCS

Fluorescence Cross-Correlation Spectroscopy

FCS

Fluorescence Correlation Spectroscopy

Field Feature

A field feature is calculated for all segmented objects of a class. The geometric or intensity parameters of all objects of the class, e.g. the area or the average intensity, are added together. In addition, all objects can be counted, for example, or the area of the objects in relation to the total image area can be calculated as a percentage.

Fluorescent beads

Fluorescent beads are often used to measure the point spread function (PSF). The diameter of these beads is usually significantly below the resolution limit of the objective used. Based on the known shape and size, various optical parameters of the microscope system can be determined with the help of such objects. To measure the PSF when using an objective with a numerical aperture of 1.4, beads with a diameter of 50-170 nm should be used.

Fourier Filter

The processed result ApoTome image can sometimes still show fine residual line artifacts. This is due to sample, staining, dynamic range during acquisition or exposure time used. These artifacts can be removed using the Fourier Filter option. This function makes use of the fact, that stripe artifacts appear as a group of dots in frequency space. The filter masks those dots and can in this way remove stripe artifacts from the image. It only works however, if the camera had been aligned parallel to the grid lines during phase calibration. Also, it is quite a crude method and should not be needed if all other preconditions for using the ApoTome have been met.

Gamma value

The gamma value makes it possible to correct the display of images on computer screens which do not allow the linear display of gray value curves. By changing the gamma value you can emphasize certain intensity ranges within your image when it is displayed on the screen. A value <1 emphasizes the ranges of medium pixel intensity (medium gray values), while a value >1 emphasizes the dark and bright pixel intensities and therefore increases the contrast. The recommended settings are 0.8 for fluorescence images, 1.2 for phase contrast or DIC images and 0.45 for true color images. Please bear in mind that a "correct" gamma value setting depends on numerous parameters, such as screen settings, ambient brightness, etc., and a universal setting cannot therefore be given.

Gaussian Distribution

The emission of fluorescent light in fact follows a Poisson distribution. If, however, detector noise predominates during imaging, or the image data are only just above the camera noise and therefore very dark, a normal distribution according to Gauss tends to apply to such images.

Generalized Cross Validation (GCV)

Regularization, which lessens the influence of noise during restoration, is normally controlled by a parameter that in most cases is determined heuristically via trial and error. The "generalized cross validation" (GCV) method makes it possible to estimate this parameter even under the complex conditions of Poisson maximum likelihood minimization.

Image Analysis Wizard

Using the Image Analysis Wizard you can create automatic measurement routines very easily. The wizard delivers precise results without any need to spend time on programming. This allows you to complete even complex measurement tasks in just a few minutes.

Image display

A maximum range of 256 gray levels (black and white image) or 16 million colors can normally be displayed on a screen. Modern digital cameras capture a much larger range: black and white cameras up to 65536 values and color cameras theoretically up to (65536)3 colors (281 billion). The display of these gray values/colors therefore needs to be adjusted for the monitor by the user. For this adjustment an upper and a lower gray/color value are defined. All gray/color values between these limits are displayed on the monitor within the 256 gray values/16 million colors that can be represented.

Image Normalization

The processing algorithm for ApoTome raw images acts in a subtractive manner effectively reducing the gray value range of the output image. Since all calculations are done internally in the high precision 32 bit floating point image format the normalize option can help to generate better output data. The floating point numbers are back-converted into 16 bit integer numbers using the full 16 bit dynamic range normalized to the brightest pixel. This option makes it impossible however to make quantitative comparisons between images.

Kymograph

A Kymograph is a twodimensional representation of a moving object over time. The movement of the object is traced using a line or curve of a given thickness and an intensity plot is then generated over time. The kymograph image displays the intensities along the line in X direction and the time points are plotted in the Y direction. With this method one can visualize and

analyze speed and acceleration of moving objects with a simple 2D representation.

Lamp Flicker

This phenomenon mainly occurs if fluorescent arc lamps are operated for a long period of time. Under certain circumstances alternating darker and brighter layers can then appear in the Z direction in Z-stacks. This effect may prevent 3D deconvolution from being usefully applied, for example.

Maximum mode

In the case of a maximum intensity projection, only the pixels with the highest intensity are displayed along the observation axis. This view is well suited to the two-dimensional display of three-dimensional images, e.g. in publications, one reason being that a maximum transparency effect is only visible in this mode.

Microscanning

Microscanning is a technological process for the production of high-resolution images using a CCD or CMOS sensor. For a sequence of images, the sensor is moved in two dimensions by micro-mechanics in very small intervals between acquisitions.The distances are smaller than a pixel dimension and allow the inclusion of detailed information that would otherwise not be seen by the sensor.

Mixed mode

In Mixed mode, a volume can be displayed in both Surface mode and Transparency mode. In the case of multichannel images, for example, structures inside a cell, such as FISH signals or nucleoli, can be displayed in Surface mode and the cytoplasm around these structures can be displayed transparently in another channel. This means that even highly complex spatial relationships can be shown convincingly.

Motif buttons

With the Motif buttons you can optimize image acquisition regarding particular requirements like speed or quality. All parameters e.g. camera resolution or dynamic range in Acquisition Mode or Channels tool were set automatically. They will influence basically camera, detector and lightning settings.

MTB

The software MicroToolBox (MTB) is used to generate and manage microscope configurations. Information about microscope components (e.g. nosepieces, reflector turrets, shutters etc.) and, if necessary, additional external units (e.g. motorized xy stages, external light sources etc.) is stored in these configurations. Furthermore, the software can also be used to enter information about microscope components, such as objectives, fluorescence filter cubes etc., in a simple way and to save this information in the microscope (depending on the type of microscope in question). In this case, the information is saved directly in the microscope, allowing it to be displayed on the microscope’s TFT screen, for example. Various configurations can be created, of which only one is activated at any time. The active configuration is used by imaging software such as ZEN to provide graphic control dialogs for the configured microscope units (e.g. light-path or microscope components control).

NDD

Non-descanned detector. The non-descanned detection modules can be used in the reflective or transmissive light path (transmission NDDs not available for Axio Imager) or simultaneously in both beam paths. That means that up to 12 NDD channels (depending on the stand used) can be configured.

Nearest Neighbor

The Nearest Neighbor method uses the simplest and fastest algorithm (Castleman, K.R., Digital Image Processing, Prentice-Hall, 1979). Its function is based on subtraction of the out-of-focus information in each plane of a stack, taking the neighboring sections above and below the corrected Z-plane into account. This method is applied sequentially to each plane of the entire 3D stack. It allows you to enhance contrast quickly, even if image stacks have not been put together optimally.

Nyquist Criterion

The Nyquist criterion states that a signal must be detected with at least double precision in order to reliably acquire all the frequencies in the signal. In the case of images acquired with coarser resolution, undesired effects such as aliasing may otherwise result. For the deconvolution of microscope images, this means, in practical terms, that images should be acquired with a pixel resolution that is at least double the optical resolution, both in the lateral and axial direction.

Object Feature

An object feature is calculated for an individual segmented object. It describes a geometric or intensity property of the object, e.g. its area or its average intensity.

PCH

Photon Counting Histogramm

Phase Correction

The ApoTome grid is moved in precise steps during acquisition in order to cover the sample fully for one section. The used steps are stored in the image metadata and used for all subsequent processing steps. In most cases this will work very well. However, there can be cases e.g. caused by vibrations, when the actual grid position deviates from the reported position causing artifacts during processing. Phase correction analyses the actual grid positions in the raw images and uses the determined values instead for processing. This option will add a bit of processing time however.

Point Spread Function (PSF)

All optoelectronic effects that influence the creation of a microscope image can be described mathematically in the form of the point spread function (PSF). If the PSF is known, deconvolution can be used to largely remove the negative effects from microscope images. There are three possible ways to determine the PSF: theoretically through knowledge of the key optical parameters, experimentally through measurement using fluorescent beads of a known diameter, or blindly using a method that works with less prior knowledge. In ZEN the theoretical model according to Lanni and Gibson has been implemented, which also models asymmetries like those that can arise due to spherical aberrations (see S. F. Gibson, F. Lanni, "Experimental test of an analytical model of aberration in an oil-immersion objective lens used in three-dimensional light microscopy", J. Opt. Soc. Am. A, vol. 8, no. 10, pp. 1601-1613, October 1991).

Poisson Distribution

The emission of photons by fluorochromes follows a statistical distribution, known as a Poisson distribution. This is the preferred model taken as the basis for the deconvolution calculation. It applies if the predominant proportion of image noise is caused by shot noise ("salt and pepper noise"). This assumption applies to images that have been acquired using good, low-noise detectors, the dynamic range of which has been utilized to a certain extent.

Position

In a tile experiment positions refer to independent individual image fields (tiles) that are localized at various places on the sample. A position corresponds to a tile region consisting of just one tile. Each position is based on an X and Y coordinate of the stage and a Z coordinate of the focus drive. Individual positions or position arrays (grouped individual positions) are defined using the Tiles tool. After acquisition the individual positions are displayed as scenes.

Pseudo color assignment

In fluorescence microscopy, pseudo color assignment describes the assignment of any artificially selected color to the channel of a multichannel fluorescence image. As it is mostly monochrome cameras (which produce black and white images rather than "true colors") that are used in this area of application, we talk of pseudo coloring.

Raw Data Mode

The ApoTome combines the advantages of widefield imaging systems with the advantages of optical sectioning. Images acquired from the Acquisition tab always contain all images acquired from the grid. These grid images are also called phase- or raw-images. This principle offers several advantages: 1) all informations acquired are kept and not discarded; 2) the acquisition itself is not slowed down by processing overhead; 3) you get access to various correction methods giving you flexibility in treating your sample in the right way after acquisition; 4) Phase (=grid-position) errors occuring during acquisition such as caused by vibrations of the microscope can be likely corrected using the phase correction option without having to redo the acquisition; 5) you can achieve a marked improvement in resolution and contrast by using the specially adapted ApoTome deconvolution option bundled with all systems; 6) the raw mode facilitates easy analysis of images which show errors or artifacts in the sectioned image which would otherwise remain obscure.

Reference Z-Position

By default the current Z-position at the time the experiment is started is set as the Reference Z-Position for acquisition. Z-stack experiments, for which the center of the defined Z-stack is set by default as the fixed Reference Z-Position, form an exception to this. Offsets for channels and Z-stacks shift acquisition in relation to the Reference Z-Position. If a focus strategy is used, this determines and updates the Reference Z-Position during the experiment.

Regularization

Working with real microscope images that are affected by noise leads to considerable difficulties with the practical application of deconvolution methods, which is why regularization (e.g. according to Tikhonov-Miller-Phillips) is essential. Regularization is a method that lessens the influence of noise by means of various penalty terms. Stronger regularization leads to weaker restoration and weaker regularization to stronger restoration, although in this case noise is also intensified.

Regularized Inverse Filter

The inverse filter is a genuine 3D method and generally achieves better results than the Nearest Neighbor algorithm. It essentially involves dividing the Fourier transformation ("FT") of the volume by the FT of the PSF, which can be performed very quickly. In the real space this corresponds to deconvolution. In addition, a statistical method ("General Cross Validation – GCV") is applied, which determines the noise component of the image and automatically sets the restoration strength to the optimum level in line with this. This process is also known as regularization. The

method is very well suited to the processing of several image stacks in order to preselect images for the application of the iterative "high-end" method. Z-stacks must, however, have been acquired at the correct (Nyquist) distance. The additional acquisition of Z-planes above and below the structure of interest is recommended.

Render Series

To display a 3D volume on the screen, each image must be recalculated. This takes time and, in the case of large images, cannot be done interactively. You can, however, have a series of individual images calculated which represent the animation that you want. Such an image series can be displayed considerably faster and more fluidly than is possible interactively on the screen as, in this case, the views no longer have to be rerendered. Furthermore, an image series like this lends itself extremely well to being exported as a film.

Shading Correction

The Brightness of microscopic images often declines to the edges. This is caused for example, by misaligned, or inhomogeneous lighting, inconstant light conditions or dirty optics. ZEN is able to correct this interference with the so called Shading Correction. First you need a white image. This functions as a reference for the background of your image, which shall be corrected.

Smart Setup

Smart Setup is the intelligent and convenient control center for your fluorescence images. Simply select a fluorochrome from the more than 500 dyes stored and ZEN will automatically provide the optimal filter combinations and acquisition settings for your experiment.

Spherical Aberration

Every objective requires the use of a defined immersion medium to deliver the best optical resolution. In microscopy practice, particularly in the area of biosciences, it is not always possible, however, to embed the sample in a medium with the correct refractive index. When light enters the embedding medium with the wrong refractive index this results in "spherical aberration". The PSF becomes more asymmetrical the further away from the cover slip it is measured. In practical terms this becomes noticeable in the form of an increasing loss of brightness as the distance from the cover slip increases. It is possible to compensate for spherical aberration either by using objectives with correction rings or objectives that have been calculated for certain embedding media (e.g. aqueous solutions). Within certain limits, however, spherical aberration can also be compensated for during deconvolution, by taking the parameters responsible for this effect into consideration when calculating the theoretical PSF. For further details see S. F. Gibson, F. Lanni, "Experimental test of an analytical model of aberration in an oil-immersion objective lens used in three-dimensional light microscopy", J. Opt. Soc. Am. A, vol. 8, no. 10, pp. 1601-1613, October 1991.

Surface mode

The two modes previously described display the data with soft transitions or with a transparent character, depending on the setting. In Surface mode, the program calculates solid surfaces ("isosurfaces") from the gray values, which emphasizes particularly flat structures (e.g. cell walls of plant cells). This display can be used if you want to draw attention to certain structures, while other, internal structures are hidden.

Threshold

Which threshold is the correct one is a question that is frequently asked. Unfortunately it is not possible to give a definitive answer to this question, particularly because this often depends on the problem and the properties of the sample. Generally speaking it can only be said that the best approach is to determine the thresholds using appropriate control samples, e.g. samples without colocalization as a negative control and samples with biologically relevant colocalization as a positive control. Thresholds determined in this way can, under certain circumstances, be transferred to the sample of interest.

Tile region

In a tile experiment a tile region refers to a group of individual image fields (tiles) that belong together and are arranged in the form of a grid. With the help of tile regions it is possible to acquire areas with dimensions that exceed the size of an individual image field. Within an experiment a number of tile regions can be acquired at various positions on the sample. Each tile region is based on an X and Y coordinate of the stage and a Z coordinate of the focus drive. Tile regions are defined using the Tiles tool. After acquisition the individual tile regions are displayed as scenes.

Transparency mode

In the Transparency mode, the structures in the image are rendered in a similar fashion as in the Volume mode. The key difference is an applied edge enhancement filter to allow more focus on relevant structures within the data while simultaneously fading out homogenous and less important areas.

Volume mode

In the Volume mode, the structures in the image are rendered as three-dimensional objects and illuminated by means of a virtual light source. Additionally, the transparency of the structures can be adjusted to allow deeper insights into the image. This allows a realistic representation of the structures in the image imitating real-life observations. It also allows a realistic and, in contrast to the maximum projection mode, a quantitative display of the volume.

Widefield

Classical microscopes frequently are called "widefield" microscopes in order to distinguish them from microscope systems with optical sectioning capability such as laser scanning microscopes. In contrast to such systems widefield microscopes do not possess the ability to discriminate betwee image information in the axial (=Z) direction leading to blurred images and therefore are only poor 3D imaging systems per se. There are methods to add this missing axial sectioning ability to widefield microscoped such as 3D deconvolution or structured illumination (ApoTome, Elyra-S)

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